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Methods of Identifying Inhibitors of

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^ FATTY ACID TRANSPORT PROTEINS *^* (FATP)

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Number 09/232,201 filed January 14, 1999, which claims the benefit of U.S. Provisional Application No. 60/110,941 filed December 4, 1998; U.S. Provisional Application No. 60/093,491 filed July 20, 1998; and U.S. Provisional Application No. 60/071,374 filed January 15, 1998. The teachings of each of these referenced applications are incorporated herein by reference in their entirety.

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GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

Long chain fatty acids (LCFAs) are an important source of energy for most organisms. They also function as blood hormones, regulating key metabolic functions such as hepatic glucose production. Although LCFAs can diffuse through the hydrophobic core of the plasma membrane into cells, this nonspecific transport cannot account for the high affinity and specific transport of LCFAs exhibited by cells such as

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cardiac muscle, hepatocytes, enterocytes, and adipocytes. The molecular mechanisms of LCFA transport remains largely unknown. Identifying these mechanisms can lead to pharmaceuticals that modulate fatty acid uptake by the intestine and by other organs, thereby alleviating certain medical conditions (e.g. obesity).

5 SUMMARY OF THE INVENTION

Described herein is a diverse family of fatty acid transport proteins (FATPs) which are evolutionarily conserved; these FATPs are plasma membrane proteins which mediate transport of LCFAs across the membranes and into cells. Members of the FATP family described herein are present in a wide variety of organisms, from mycobacteria to humans, and exhibit very different expression patterns in tissues among the organisms. FATP family members are expressed in prokaryotic and eukaryotic organisms and comprise characteristic amino acid domains or sequences which are highly conserved across family members. In addition, the function of the FATP gene family is conserved throughout evolution, as shown by the fact that the *Caenorhabditis* (C). *elegans* and mycobacterial FATPs described herein facilitate LCFA uptake when they are overexpressed in COS cells or *Escherichia* (E.) *coli*, respectively. FATPs are expressed in a wide variety of tissues, including all tissues which are important to fatty acid metabolism (uptake and processing).

In specific embodiments, FATPs of the present invention are from such diverse organisms as humans (*Homo* (H.) *sapiens*), mice, (*Mus* (M.) *musculus*), *F. rubripes*, *C. elegans*, *Drosophila* (D.) *melanogaster*, *Saccharomyces* (S.) *cerevisiae*, *Aspergillus nidulans*, *Cochliobolu heterostrophus*, *Magnaporthe grisea* and *Mycobacterium* (M.), such as *M. tuberculosis*. As described herein, four novel mouse FATPs, referred to as mmFATP2, mmFATP3, mmFATP4 and mmFATP5, and six human FATPs, referred to as hsFATP1, hsFATP2, hsFATP3, hsFATP4, hsFATP5 and hsFATP6, have been identified. All four novel murine FATPs (mmFATP2-5) and a previously identified murine FATP (renamed herein FATP1) have orthologs in humans (hsFATP1-5); the

sixth human FATP (hsFATP6) does not as yet have a mouse ortholog. The expression patterns of these FATPs vary, as described in detail below.

The present invention relates to FATP family members from prokaryotes and eukaryotes, nucleic acids (DNA, RNA) encoding FATPs, and nucleic acids which are useful as probes or primers (e.g., for use in hybridization methods, amplification methods) for example, in methods of detecting FATP-encoding genes, producing FATPs, and purifying or isolating FATP-encoding DNA or RNA. Also the subject of this invention are antibodies (polyclonal or monoclonal) which bind an FATP or FATPs; methods of identifying additional FATP family members (for example, orthologs of those FATPs described herein by amino acid sequence) and variant alleles of known FATP genes; methods of identifying compounds which bind to an FATP, or modulate or alter (enhance or inhibit) FATP function; compounds which modulate or alter FATP function; methods of modulating or altering (enhancing or inhibiting) FATP function and, thus, LCFA uptake into tissues of a mammal (e.g. human) by administering a compound or molecule (a drug or agent) which increases or reduces FATP activity; and methods of targeting compounds to tissues by administering a complex of the compound to be targeted to tissues and a component which is bound by an FATP present on cells of the tissues to which the compound is to be targeted. For example, a complex of a drug to be delivered to the liver and a component which is bound by an FATP present on liver cells (e.g., FATP5) can be administered.

In one embodiment, the present invention relates to modulating or altering (enhancing or inhibiting/reducing) LCFA uptake in the small intestine and, thus, increasing or reducing the number of calories in the form of fats available to an individual. In another embodiment, the present invention relates to inhibiting or
25 reducing LCFA uptake in the small intestine in order to reduce circulating fatty acid levels; that is, LCFA uptake in the small intestine is reduced and, therefore, circulating (blood) levels are not as high as they otherwise would be. FATP4 has been shown to be expressed in epithelial cells of the small intestine and particularly in the brush border layer of the small intestine. FATP2 has also been shown to be expressed at low levels

in epithelial cells of the small intestine, particularly in the duodenum. In contrast, FATP1, FATP3, FATP5 and FATP6 were not detected in any of the intestinal tissues. Thus, also described herein are FATPs which are present in the epithelial cell layer of the small intestine where they mediate LCFA uptake. These FATPs, particularly

5 FATP4 and also FATP2, are targets for methods and drugs which block their function or activity and are useful in treating obesity, diabetes and heart disease. The ability of these FATPs to mediate fat uptake can be modulated or altered (enhanced or inhibited), thus modulating fat uptake in the small intestine. This can be done, for example, by administering to an individual, such as a human or other animal, a drug which blocks

10 interaction of LCFAs with FATP4 and/or FATP2 in the small intestine, thus inhibiting LCFA passage into the cells of the small intestine. As a result, fat absorption is reduced and, although the individual has consumed a certain quantity of fat, the LCFAs are not absorbed to the same extent they would have been in the absence of the compound administered.

15 Thus, one embodiment of this invention is a method of reducing LCFA uptake (absorption) in the small intestine and, as a result, reducing caloric uptake in the form of fat. A further embodiment is a compound (drug) useful in inhibiting or reducing fat absorption in the small intestine. In another embodiment, the invention is a method of reducing circulating fatty acid levels by administering to an individual a compound

20 which blocks interactions of LCFAs with FATP4 and/or FATP2 in the small intestine, thus inhibiting LCFA passage into cells of the small intestine. As a result, fatty acids pass into the circulatory system at a diminished level and/or rate, and circulating fatty acid levels are lower than they would be in the absence of the compound administered. This method is particularly useful for therapy in individuals who are at risk for or have

25 hyperlipidemia. That is, it can be used to prevent the occurrence of elevated levels of lipids in the blood or to treat an individual in whom blood lipid levels are elevated. Also the subject of this invention is a method of identifying compounds which alter FATP function (and thus, in the case of FATP2 and/or FATP4, alter LCFA uptake in the small intestine).

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In another embodiment, the present invention relates to a method of modulating or altering (enhancing or inhibiting) the function of FATP6, which is expressed at high levels in the heart. A method of inhibiting FATP6 function is useful, for example, in individuals with heart disease, such as ischemia, since reducing LCFA uptake into heart muscle in an individual who has ischemic heart disease, which may be manifested by, for example, angina or heart attack, can reduce symptoms or reduce the extent of damage caused by the ischemia. In this embodiment, a drug which inhibits FATP6 function is administered to an individual who has had or is having a heart attack, to reduce LCFA uptake by the individual's heart and, as a result, reduce the damage caused by ischemia. In a further embodiment, this invention is a method of targeting a compound, such as a therapeutic drug or an imaging reagent, to heart tissue by administering to an individual (e.g., a human) a complex of the compound and a component (e.g., a LCFA or LCFA-like compound) which is bound by an FATP (e.g., FATP6) present in cells of heart tissue.

In a further embodiment, LCFA uptake by the liver is modulated or altered (enhanced or reduced), in an individual. For example, a drug which inhibits the function of an FATP present in liver (e.g., FATP5) is administered to an individual who is diabetic, in order to reduce LCFA uptake by liver cells and, thus reduce insulin resistance.

The present invention, thus, provides methods which are useful to alter, particularly reduce, LCFA uptake in individuals and, as a result, to alter (particularly reduce), availability of the LCFAs for further metabolism. In a specific embodiment, the present invention provides methods useful to reduce LCFA uptake and, thus, fatty acid metabolism in individuals, with the result that caloric availability from fats is reduced, and circulating fatty acid levels are lower than they otherwise would be. These methods are useful, for example, as a means of weight control in individuals, (e.g., humans) and as a means of preventing elevated serum lipid levels or reducing serum lipid levels in humans. FATPs expressed in the small intestine, such as FATP4, are useful targets to be blocked in treating obesity (e.g., chronic obesity) or to be enhanced

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in treating conditions in which enhanced LCFA uptake is desired (e.g., malabsorption syndrome or other wasting conditions).

The identification of this evolutionarily conserved fatty acid transporter family will allow a better understanding of the mechanisms whereby LCFAs traverse the lipid bilayer as well as yield insight into the control of energy homeostasis and its dysregulation in diseases such as diabetes and obesity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence alignment of FATPs: mmFATP1 (SEQ ID NO:92), mmFATP2 (SEQ ID NO:93), mmFATP3 (SEQ ID NO:94), mmFATP4 (SEQ ID NO:95), mmFATP5 (SEQ ID NO:96), ceFATPa (SEQ ID NO:97), scFATP (SEQ ID NO:98) and mtFATP (SEQ ID NO:99). The underlining (amino acid residues 204-212 of mtFATP) indicates an AMP binding motif which is found in many classes of proteins; the underlining at amino acid residues 204-507 of the mtFATP sequence indicates the FATP 360 amino acid signature sequence.

Figures 2A-2D show results of LCFA uptake assays. Figures 2A-2D: COS cells were cotransfected using the DEAE-dextran method with the mammalian expression vectors pCDNA-CD2 either alone (control; Figure 2A) or in combination with one of the FATP-containing expression vectors (pCDNA-mmFATP1, Figure 2B; pCDNA-mmFATP2, Figure 2C; or pCMV-SPORT2-mmFATP5, Figure 2D) as described in Materials and Methods for Example 2. COS cells were gated on forward scatter (FSC) and side scatter (SS), and the results shown represent >10,000 cells. Cells exhibiting >300 CD2 fluorescence units (vertical line) representing 15% of all cells were deemed CD2 positive.

Figure 3 is a graph of fluorescence of cells expressing a FATP gene. As in Figures 2A-2D, COS cells were cotransfected with pCDNA-CD2 either alone (control) or in combination with one of the FATP-containing expression vectors (pCDNA-mmFATP1, pCDNA-mmFATP2, pCMV-SPORT2-mmFATP5, or pCDNA-ceFATPb). The mean BODIPY-FA fluorescence of the CD2-positive cells is plotted; results shown

represent the average of three experiments, each consisting of greater than 50,000 COS cells. Note that a logarithmic scale is used on the ordinate.

Figure 4 is a graph of the uptake of palmitate with time. The full-length coding region of mtFATP (squares) or a control protein (TFE3; circles) was subcloned into the inducible, prokaryotic expression vector pET (Novagen). Expression from the resulting plasmid was induced (solid symbols) in transformed *E. coli* cells with 1 mM isopropyl- β -D-thiogalactoside (IPTG) for 1 hour, or cells were left uninduced (open symbols). Data points were done in triplicate and counts were normalized to the number of bacteria as determined by OD₆₀₀.

Figure 5 is a phylogenetic tree produced by aligning complete and partial sequences for *FATP* genes from human, rat, mouse, puffer fish, *D. melanogaster*, *C. elegans*, *S. cerevisiae*, and *M. tuberculosis* using ClustalX and using these data to produce a phylogenetic tree using TreeViewPPC. The bar indicates the number of substitutions per residue, i.e., 0.1 corresponds to a distance of 10 substitutions per 100 residues.

Figure 6 shows a comparison of the FATP signature sequences of mmFATP1 (SEQ ID NO:1), mmFATP5, (SEQ ID NO:2), ceFATPa (SEQ ID NO:3), scFATP (SEQ ID NO:4) and mtFATP (SEQ ID NO:5).

Figure 7 shows the sequence identity among the FATP family members and VLACs, based on the 360 amino acid signature sequence of FATP from Figure 1.

Figures 8A and 8B are the mmFATP3 DNA sequence (SEQ ID NO:6).

Figure 9 is the mmFATP3 protein sequence (SEQ ID NO:7).

Figures 10A and 10B are the mmFATP4 DNA sequence (SEQ ID NO:8).

Figure 11 is the mmFATP4 protein sequence (SEQ ID NO:9).

Figures 12A and 12B are the mmFATP5 DNA sequence (SEQ ID NO:10).

Figure 13 is the mmFATP5 protein sequence (SEQ ID NO:11).

Figures 14A and 14B are the hsFATP2 DNA sequence (SEQ ID NO:12).

Figure 15 is the hsFATP2 protein sequence (SEQ ID NO:13).

Figures 16A and 16B are the hsFATP3 DNA sequence (SEQ ID NO:14).

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Figure 19 is the hsFATP4 protein sequence (SEQ ID NO:17).

Figure 21 is the hsFATP5 protein sequence (SEQ ID NO:19).

Figure 23 is the hsFATP6 protein sequence (SEQ ID NO:21).

Figure 25 is the mtFATP protein sequence (SEQ ID NO:23).

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Figure 27 shows the DNA sequence (SEQ ID NO:26) and predicted amino acid sequence (SEQ ID NO:27) of human FATP4.

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Figure 28B is the amino acid composition of hsFATP1.

Figure 28C is a hydrophilicity plot for hsFATP1, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues at a time.

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Figure 29B is a listing of the amino acid composition of hsFATP4.

Figure 29C is a hydrophilicity plot for hsFATP4, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues at a time.

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Figures 31A and 31B show a comparison of the nucleotide sequence of human FATP4 (SEQ ID NO:30) and the nucleotide sequence of mouse FATP4 (SEQ ID NO:31).

Figure 32 shows a comparison of the amino acid sequence of human FATP1 (SEQ ID NO:32) and the amino acid sequence of mouse FATP1 (SEQ ID NO:33). Shaded amino acid residues match the consensus sequence exactly

Figure 33 shows a comparison at the amino acid level of human FATP4 (SEQ ID NO:34) and mouse FATP4 (SEQ ID NO:35). Shaded amino acid residues match the consensus sequence exactly.

Figure 34 shows the nucleotide sequence (SEQ ID NO:36) and predicted amino acid sequence (SEQ ID NO:37) of hsFATP6.

Figure 35A is a hydrophobicity plot for hsFATP6, showing that it has multiple membrane-spanning domains.

Figure 35B is a listing of the amino acid composition of hsFATP6.

Figure 35C is a hydrophilicity plot for hsFATP6, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues at a time.

Figure 36 shows an alignment of the amino acid sequences of hsFATP1 (SEQ ID NO:38), hsFATP4 (SEQ ID NO:39) and hsFATP6 (SEQ ID NO:40). Shaded amino acid residues match the consensus sequence exactly.

Figure 37 shows results of assessment of fatty acid uptake by human FATP1 and human FATP4. The percent of CD2-positive cells exhibiting a BODIPY-fluorescence of more than 300 arbitrary units is plotted for the three different conditions tested.

Figure 38 is a graph showing uptake of tritiated oleate, with time, by 293 cells transfected with either (diamonds) a plasmid for expression of human FATP4 or (squares) a control plasmid.

Figure 39 is an illustration of the amino acid sequences of human FATP4 (SEQ ID NO:41) and mouse FATP4 (SEQ ID NO:42) compared to human FATP1 (SEQ ID NO:43). Shown by underlining are the FATP consensus sequence (236-556 of hsFATP1) and the AMP-binding motif (246-254 of hsFATP1). The human FATPs were cloned by screening libraries with sequences from ESTs (expressed sequence tags). Mouse FATP4 was cloned by PCR using degenerate primers.

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Figure 40 is a graph showing the uptake, with time, of tritiated oleate by mouse enterocytes in the presence of no oligonucleotide (squares), sense oligonucleotide (circles) or antisense oligonucleotide (diamonds).

Figure 41 is a bar graph showing uptake of tritiated oleate, by mouse enterocytes
5 in the presence of various concentrations of antisense (solid bars), mismatch (stippled bars) or sense (lined bars) oligonucleotides.

Figure 42 is a bar graph showing uptake of tritiated oleate and uptake of ³⁵S-labeled methionine by mouse enterocytes to which were added no oligonucleotide, the antisense oligonucleotide, or the mismatch oligonucleotide.

10 Figure 43A is the nucleotide sequence of the gene encoding mouse FATP4 (SEQ ID NO:44).

Figure 43B is the amino acid sequence of mouse FATP4 protein (SEQ ID NO:45).

Figures 44A, 44B, and 44C are the hsFATP1 DNA sequence (SEQ ID NO:46).
15 Coding region: 175-2115 (1941 nt).

Figure 45 is the hsFATP1 protein sequence (SEQ ID NO:47).

Figures 46A and 46B are the hsFATP2 DNA sequence (SEQ ID NO:48).
Coding region: 223-2085 (1863 nt).

Figure 47 is the hsFATP2 protein sequence (SEQ ID NO:49).

20 Figure 48 is the partial DNA sequence of hsFATP3 (SEQ ID NO:50). Coding region: 1-993.

Figure 49 is the partial protein sequence of hsFATP3 (SEQ ID NO:51).

Figures 50A, 50B, and 50C are the hsFATP4 DNA sequence (SEQ ID NO:52).
Coding region: 208-2139 (1932 nt).

25 Figure 51 is the hsFATP4 protein sequence (SEQ ID NO:53).

Figure 52 is the hsFATP5 partial DNA sequence (SEQ ID NO:54). Coding region: 1-1062.

Figure 53 is the hsFATP5 partial protein sequence (SEQ ID NO:55).

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Figures 54A, 54B, and 54C are the hsFATP6 DNA sequence (SEQ ID NO:56).
Coding region: 643-2502 (1860 nt).

Figure 55 is the hsFATP6 protein sequence (SEQ ID NO:57).

Figures 56A, 56B, and 56C are the rnFATP1 DNA sequence (rn=*Rattus*
5 *norvegicus*; (SEQ ID NO:58). Coding region: 75-2015 (1941 nt).

Figure 57 is the rnFATP1 protein sequence (SEQ ID NO:59).

Figure 58A, 58B, and 58C are the rnFATP2 DNA sequence (SEQ ID NO:60).
Coding region: 795-2657 (1863 nt).

Figure 59 is the rnFATP2 protein sequence (SEQ ID NO:61).

10 Figure 60A and 60B are the rnFATP4 partial DNA sequence (SEQ ID NO:62).
Coding region: 1-1218.

Figure 61 is the rnFATP4 partial DNA sequence (SEQ ID NO:63).

Figure 62A, 62B, and 62C are the mmFATP1 DNA sequence (SEQ ID NO:64).
Coding region: 1-1944.

15 Figure 63 is the mmFATP1 protein sequence (SEQ ID NO:65).

Figures 64A and 64B are the mmFATP2 DNA sequence (SEQ ID NO:66).
Coding region: 121-1992 (1872 nt).

Figure 65 is the mmFATP2 protein sequence (SEQ ID NO:67).

Figures 66A and 66B are the mmFATP3 partial DNA sequence (SEQ ID
20 NO:68). Coding region: 1-1830.

Figure 67 is the mmFATP3 partial protein sequence (SEQ ID NO:69).

Figures 68A, 68B, and 68C are the mmFATP4 DNA sequence (SEQ ID NO:70).
Coding region: 1-1932.

Figures 69 is the mmFATP4 protein sequence (SEQ ID NO:71).

25 Figures 70A and 70B are the mmFATP5 DNA sequence (SEQ ID NO:72).
Coding region: 60-2129.

Figure 71 is the mmFATP5 protein sequence (SEQ ID NO:73).

Figures 72A and 72B are the dmFATP partial DNA sequence (dm=*Drosophila*
melanogaster; SEQ ID NO:74). Coding region: 1-1773.

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Figures 73 is the dmFATP partial protein sequence (SEQ ID NO:75).

Figure 74 is the drFATP partial DNA sequence (dr=*Danio rerio*, zebrafish; SEQ ID NO:76) Coding region: 1-173.

Figure 75 is the drFATP partial protein sequence (SEQ ID NO:77).

5 Figure 76A and 76B are the ceFATPa DNA sequence (SEQ ID NO:78). Coding region: 1-1953.

Figure 77 is the ceFATPa protein sequence (SEQ ID NO:79).

Figures 78A and 78B are the ceFATPb DNA sequence (SEQ ID NO:80). Coding region: 1-1968.

10 Figure 79 is the ceFATPb protein sequence (SEQ ID NO:81).

Figures 80A and 80B are the chFATP DNA sequence (SEQ ID NO:82; ch=*Cochliobolu heterostrophus*). Coding region: 1-1932.

Figure 81 is the chFATP protein sequence (SEQ ID NO:83).

Figure 82 is the anFATP partial protein sequence (an=*Aspergillus nidulans*; 15 SEQ ID NO:84). Coding region: 1-597.

Figure 83 is the anFATP partial protein sequence (SEQ ID NO:85).

Figure 84 is the mgFATP partial DNA sequence (mg=*Magnaporthe grisea*, rice blast; SEQ ID NO:86). Coding region: 1-522.

Figure 85 is the mgFATP partial protein sequence (SEQ ID NO:87).

20 Figures 86A and 86B are the scFATP DNA sequence (SEQ ID NO:88). Coding region: 1-1872.

Figure 87 is the scFATP protein sequence (SEQ ID NO:89).

Figures 88A and 88B are the mtFATP DNA sequence (SEQ ID NO:90).

Figure 89 is the mtFATP protein sequence (SEQ ID NO:91). Coding region: 1- 25 1794.

Figure 90 is a consensus sequence of the FATP signature sequence (SEQ ID NO:100), based on 23 independent sequences aligned in ClustalX. The height of the bar at each amino acid residue position indicates the degree of conservation at that position. Gaps have been inserted to maintain the strength of the alignment.

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Figure 91 is a hydrophilicity plot for hsFATP2, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues at a time.

Figure 92 is a hydrophilicity plot for the hsFATP3 partial protein, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues
5 at a time.

Figure 93 is a hydrophilicity plot for the hsFATP5 partial protein, made using the Kyte-Doolittle method, averaging hydrophilicity values for 18 amino acid residues at a time.

Figures 94A and 94B are a representation of the DNA sequence (SEQ ID
10 NO:101) of the hsFATP3 gene, and the amino acid sequence (SEQ ID NO:102) of the hsFATP3 protein.

Figure 95. Mammalian expression constructs containing either hsFATP4 (squares and triangles) or empty control vector (circles) were stably transfected into 293 cells. Short-term uptake of Bodipy-FA in the presence of BSA was determined by
15 FACS. The mean fluorescence of the viable cell population is expressed in arbitrary fluorescence units. FATP4 protein expression was determined by densitometry of anti-FATP4 Western blots, and is expressed in arbitrary units.

Figure 96. Short-term uptake of Bodipy-palmitate (1 μ M), either by control cells (black bars) or FATP4-expressing cells (hatched bars), was measured in the
20 presence of 0, 10, 100 μ M unlabeled palmitate. FA uptake was quantified by FACS and expressed in arbitrary fluorescence units.

Figure 97. The rate of [3 H]palmitate uptake by 293 cells, which were stably transfected with a construct for either human FATP4 (diamonds) or an empty vector (circles), was compared to that of isolated enterocytes (squares).

25 Figure 98. Isolated enterocytes were incubated for 48h with increasing concentrations of the FATP4 antisense oligonucleotide or with 100 μ M of a randomized control oligonucleotide with identical nucleotide composition to the FATP4 antisense oligonucleotide. The uptake of oleate by the enterocytes was then measured over a 5 min time interval (solid bars). In parallel, the levels of FATP4 protein and, as a loading

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control, β -catenin, were determined by Western blotting and quantitated using densitometry (hatched bars). FA uptake and FATP4 protein levels were normalized to that of untreated cells. The averages and standard deviations of 4 independent experiments are shown.

Figure 99. Uptake rates of [^3H]oleate, [^3H]palmitate and [^{35}S]methionine by primary enterocytes were measured after 48h incubation with either 100 μM FATP4 antisense (solid bars) or 100 μM randomized control oligonucleotide (hatched bars) and expressed as % of untreated cells.

DETAILED DESCRIPTION OF THE INVENTION

As described herein, FATPs are a large evolutionarily conserved family of proteins that mediate the transport of LCFAs into cells. The family includes proteins which are conserved from mycobacteria to humans and exhibit very different expression patterns in tissues. Specific embodiments described include FATPs from mice, humans, nematodes, fungi and mycobacteria which have been shown to be functional LCFA transporters. The term "fatty acid transport proteins" ("FATPs") as used herein, refers to the proteins described herein as FATP1, FATP2, FATP3, FATP4, FATP5 and FATP6, which have been described in one or more species of mammals, as well as mtFATP, ceFATP, scFATP, anFATP, mgFATP, and chFATP, and other proteins sharing at least about 50% amino acid sequence similarity, preferably at least about 60% sequence similarity, more preferably at least about 70% sequence similarity, and still more preferably, at least about 80% sequence similarity, and most preferably, at least about 90% sequence similarity in the approximately 360 amino acid signature sequence. The approximately 360 amino acid FATP signature sequence is shown in Figure 1. The consensus sequence of the signature sequence is shown in Figure 90. The nomenclature used herein to refer to FATPs includes a species-specific prefix (e.g., mm, *Mus musculus*; hs or h, *Homo sapiens* or human; mt *M. tuberculosis*; dm. *D. melanogaster*; ce, *C. elegans*; sc, *Saccharomyces cerevisiae*) and a number such that mammalian homologues in different species share the same number. For example, six human and

five mouse *FATP* genes which are expressed in a variety of tissues are described herein and are referred to, respectively, as hsFATP1-hsFATP6 and mmFATP1-mmFATP5; for example, hsFATP4 and mmFATP4 are the human and mouse orthologs.

Expression patterns of human and mouse FATPs have been assessed and are described below. Briefly, results of these assessments show that FATP5 is a liver-specific gene. FATP2 is highly expressed in liver and kidney. Both of these proteins, as well as FATP4 and FATPs from nematodes and mycobacteria, have been shown to be functional LCFA transporters. Results have also shown that FATP4 mRNA is present at high levels in epithelial cells of two regions of the small intestine (the jejunum and ileum) and at lower, but significant, levels in a third region (the duodenum). They further showed that FATP2 mRNA is present in epithelial cells of the duodenum at a level similar to that of FATP4 mRNA levels, but is present at lower levels in the jejunum and ileum. FATP4 mRNA was absent from other cell types of the small intestine and no FATP4 mRNA could be detected in any cells of the colon. No signals above background could be detected for FATP1, FATP3 and FATP5 in any of the intestinal tissues. Thus, FATP4 is the major FATP in the mouse small intestine, which supports a major role for FATP4 (along with FATP2 to a lesser extent) in absorption of free fatty acids. hsFATP4 was clearly expressed in the jejunum and ileum; expression was absent in the stomach. This, too, is consistent with a major role for FATP4 in absorption of fatty acids in the human gut. Analysis of FATP expression in human tissues, also described in detail below, showed that hsFATP6, which has no mouse ortholog as yet, is expressed at high levels in the heart and at low levels in the placenta, but is undetectable in the other tissues assessed (Example 9). This is consistent with a major role for FATP6 in absorption of fatty acids in the heart.

Long chain fatty acids (LCFAs) are an important energy source for pro- and eukaryotes and are involved in diverse cellular processes, such as membrane synthesis, intracellular signaling, protein modification, and transcriptional regulation. In developed Western countries, human dietary lipids are mainly di- and triglycerides and account for approximately 40% of caloric intake (Weisburger, J. H. (1997) *J. Am. Diet.*

- Assoc.* 97:S16-S23). These lipids are broken down into fatty acids and glycerol by pancreatic lipases in the small intestine (Chapus, C., Rovey, M., Sarda, L & Verger, R. (1988) *Biochimie* 70:1223-34); LCFAs are then transported into brush border cells, where the majority is re-esterified and secreted into the lymphatic system as
- 5 chylomicrons (Green, P.H. & Riley, J.W. (1981) *Aust. N.Z.J. Med.* 11:84-90). Fatty acids are liberated from lipoproteins by the enzyme lipoprotein lipase, which is bound to the luminal side of endothelial cells (Scow, R.O. & Blachette-Mackie, E.J. (1992) *Mol. Cell. Biochem* 116:181-191). "Free" fatty acids in the circulation are bound to serum albumin (Spector, A.A. (1984) *Clin. Physiol. Biochem* 2:123-134) and are rapidly
- 10 incorporated by adipocytes, hepatocytes, and cardiac muscle cells. The latter derive 60-90% of their energy through the oxidation of LCFAs (Neely, J.F. Rovetto, M.J. & Oram, J.F. (1972) *Prog. Cardiovasc. Dis.* 15:289-329). Although saturable and specific uptake of LCFAs has been demonstrated for intestinal cells, hepatocytes, cardiac myocytes, and adipocytes, the molecular mechanisms of LCFA transport across the
- 15 plasma membrane have remained controversial (Hui, T.Y. & Bernlohr, D.A. (1997) *Front. Biosci.* 15:d222-31-d231; Schaffer, J.E. & Lodish, H.F. (1995) *Trends Cardiovasc. Med.* 5:218-224). Described herein is a large family of highly homologous mammalian LCFA transporters which show wide expression, including in all tissues relevant to fatty acid metabolism. Further described are novel members of this family
- 20 in other species, including mycobacterial and nematode FATPs which, like their mammalian counterparts, are functional fatty acid transporters.

The discovery of a diverse but highly homologous family of FATPs is reminiscent of the glucose transporter family. In a manner similar to the FATPs, the glucose transporters have very divergent patterns of tissue expression (McGowan, K.M.,

25 Long, S.D. & Pekala, P.H. (1995) *Pharmacol. Ther.* 66:465-505). The FATPs, like glucose transporters, may also differ in their substrate specificities, uptake kinetics, and hormonal regulation (Thorens, B. (1996) *Am. J. Physiol.* 270:G541-G553). Indeed, the levels of fatty acids in the blood, like those of glucose, can be regulated by insulin and are dysregulated in diseases such as noninsulin-dependent diabetes and obesity (Boden,

G. (1997) *Diabetes* 46:3-10). The underlying mechanisms for the regulation of free fatty acid concentrations in the blood are not understood, but could be explained by hormonal modulation of FATPs.

Insulin-resistance is thought to be the major defect in non insulin-dependent
5 diabetes mellitus (NIDDM) and is one of the earliest manifestations of NIDDM
(McGarry (1992) *Science* 258:766-770). Free fatty acids (FFAs) may provide an
explanation for why obesity is a risk factor for NIDDM. Plasma levels of FFAs are
elevated in diabetic patients (Reaven *et al.* (1988) *Diabetes* 37:1020). Elevated plasma
free fatty acids (FFAs) have been demonstrated to induce insulin-resistance in whole
10 animals and humans (Boden (1998) *Front. Biosci.* 3:D169-D175). This insulin-
resistance is likely mediated by effects of FFAs on a variety of issues. FFAs added to
adipocytes *in vitro* induce insulin resistance in this cell type as evidenced by inhibition
of insulin-induced glucose transport (Van Epps-Fung *et al.* (1997) *Endocrinology*
138:4338-4345). Rats fed a high fat diet developed skeletal muscle insulin resistance as
15 evidenced by a decrease in insulin-induced glucose uptake by skeletal muscle (Han *et*
al., (1997) *Diabetes* 46:1761-1767). In addition, elevated plasma FFAs increase
insulin-suppressed endogenous glucose production in the liver (Boden (1998) *Front.*
Biosci. 3:D169-D175), thus increasing hepatic glucose output. It has been postulated
that the adverse effects of plasma free fatty acids are due to the FFAs being taken up
20 into the cell, leading to an increase in intracellular long chain fatty acyl CoA;
intracellular long chain acyl CoAs are thought to mediate the effects of FFAs inside the
cell. Thus, fatty acid induced insulin-resistance may be prevented by blocking uptake
of FFAs into select tissues, in particular liver (by blocking FATP2 and/or FATP5),
adipocyte (by blocking FATP1), and skeletal muscle (by blocking FATP1). Blocking
25 intestinal fat absorption (by blocking FATP4) is also expected to reduce plasma FFA
levels and thus improve insulin resistance.

During the pathogenesis of NIDDM insulin-resistance can initially be
counteracted by increasing insulin output by the pancreatic beta cell. Ultimately, this
compensation fails, beta cell function decreases and overt diabetes results (McGarry

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(1992) *Science* 258: 766-770). Manipulating beta cell function is a second point where fatty acid transporter blockers may be beneficial for diabetes. While no FATP homolog has been identified so far that is expressed in the beta cell of the pancreas, the data described below suggest the existence of such a transporter and the sequence

5 information included herein provides the means to identify such a transporter by degenerate PCR, using primers to regions conserved in all FATP family members or by low stringency hybridization. It has been demonstrated that exposure of pancreatic beta-cells to FFAs increases the basal rate of insulin secretion; this in turn leads to a decrease in the intracellular stores of insulin, resulting in decreased capacity for insulin

10 secretion after chronic exposure (Bollheimer *et al.*, (1998) *J. Clin. Invest.* 101:1094-1101). The effects of FFAs are again likely to be mediated by intracellular long chain fatty acyl CoA molecules (Liu *et al.*, (1998) *J. Clin. Invest.* 101:1870-1875). FFAs have also been demonstrated to increase beta cell apoptosis (Shimabukuro *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* 95:2498-2502), possibly contributing to the decrease in beta

15 cell numbers in late stage NIDDM.

Another finding with potentially broad implications is the identification of a FATP homologue in *M. tuberculosis*. Tuberculosis causes more deaths worldwide than any other infectious agent and drug-resistant tuberculosis is re-emerging as a problem in industrialized nations (Bloom, B.R. & Small, P.M. (1998) *N. Engl. J. Med.* 338:677-

20 678). *Mycobacterium tuberculosis* has about 250 enzymes involved in fatty acid metabolism, compared with only about 50 in *E. coli*. It has been suggested that, living as a pathogen, the mycobacteria are largely lipolytic, rather than lipogenic, relying on the lipids within mammalian cells and the tubercle (Cole, S.T. *et al.*, *Nature* 393:537-544 (1998)). The *de novo* synthesis of fatty acids in *Mycobacterium leprae* is

25 insufficient to maintain growth (Wheeler, P.R., Bulmer, K & Ratledge, C. (1990) *J. Gene. Microbiol.* 136:211-217). Thus, it is reasonable to expect that inhibitors of mtFATP will serve as therapeutics for tuberculosis. FATPs expressed in mycobacteria can be targeted to reduce or prevent replication of mycobacteria (e.g., to reduce or prevent replication of *M. tuberculosis*) and, thus, reduce or prevent their adverse effects.

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For example, a FATP or FATPs expressed by *M. tuberculosis* can be targeted and inhibited, thus reducing or preventing growth of this pathogen (and tuberculosis in humans and other mammals). An inhibitor of an *M. tuberculosis* FATP can be identified, using methods described herein (e.g., expressing the FATP in an appropriate host cell, such as *E. coli* or COS cells; contacting the cells with an agent or drug to be assessed for its ability to inhibit the FATP and, as a result, mycobacterial growth, and assessing its effects on growth). A drug or agent identified in this manner can be further tested for its ability to inhibit a *M. tuberculosis* FATP and *M. tuberculosis* infection in an appropriate animal model or in humans. A method of inhibiting mycobacterial growth, particularly growth of *M. tuberculosis*, and compounds useful as drugs for doing so are also the subject of this invention.

An isolated polynucleotide encoding mtFATP, like other polynucleotides encoding FATPs of the FATP family, can be incorporated into vectors, nucleic acids of viruses, and other nucleic acid constructs that can be used in various types of host cells to produce mtFATP. This mtFATP can be used, as it appears on the surface of cells, or in various artificial membrane systems, to assess fatty acid transport function, to identify ligands and molecules that are modulators of fatty acid transport activity. Molecules found to be inhibitors of mtFATP function can be incorporated into pharmaceutical compositions to administer to a human for the treatment of tuberculosis.

Particular embodiments of the invention are polynucleotides encoding a FATP of *Cochliobolus (Helminthosporium) heterostrophus* or portions or variants thereof, the isolated or recombinantly produced FATP, methods for assessing whether an agent binds to the chFATP, and further methods for assessing the effect of an agent being tested for its ability to modulate fatty acid transport activity. *Cochliobolus heterostrophus* is an ascomycete that is the cause of southern corn leaf blight, an economically important threat to the corn crop in the United States. The related species *C. sativus* causes crown rot and common root rot in wheat and barley. One or more FATPs of *C. heterostrophus* can be targeted for the identification of an inhibitor of chFATP function, which can be then be used as an agent effective against infection of

plants by *C. heterostrophus* and related organisms. Methods described herein that were applied in studying the expression of a FATP gene and the function of the FATP in its natural site of expression or in a host cell, can be used in the study of the chFATP gene and protein.

5 *Magnaporthe grisea* (rice blast) is an economically important fungal pathogen of rice. Further embodiments of the invention are nucleic acid molecules encoding a FATP of *Magnaporthe grisea*, portions thereof, or variants thereof, isolated mgFATP, nucleic acid constructs, and engineered cells expressing mgFATP. Other aspects of the invention are assays to identify an agent which binds to mgFATP and assays to identify
10 an agent which modulates the function of mgFATP in cells in which mgFATP is expressed or in artificial membrane systems. Agents identified as inhibiting mgFATP activity can be developed into anti-fungal agents to be used to treat rice infected with rice blast.

Caenorhabditis elegans is a nematode related to plant pathogens and human
15 parasites. An isolated polynucleotide which encodes ceFATP, like other polynucleotides encoding FATPs of the FATP family described herein, can be incorporated into nucleic acid vectors and other constructs that can be used in various types of cells to produce ceFATP. ceFATP as it occurs in cells or as it can be isolated or incorporated into various artificial or reconstructed membrane systems, can be used
20 to assess fatty acid transport, and to identify ligands and agents that modulate fatty acid transport activity. Agents found by such assays to be inhibitors of ceFATP activity can be incorporated into compositions for the treatment of diseases caused by genetically related organisms with a FATP of similar sensitivity to the agents.

Aspergillus nidulans is one of a family of fungal species that can infect humans.
25 Further embodiments of the invention of the family of polynucleotides encoding FATPs are polynucleotides encoding a FATP of *Aspergillus nidulans*, and vectors and host cells that can be constructed to comprise such polynucleotides. Further embodiments are a polypeptide encoded by such polynucleotides, portions thereof having one or more functions characteristic of a FATP, and various methods. The methods include those

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5 One aspect of the invention relates to isolated nucleic acids that encode a FATP as described herein, such as those FATPs having an amino acid sequence in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:102), Figure 51 (SEQ ID NO:53), Figure 53 (SEQ ID NO:55), and Figure 55 (SEQ ID NO:57) and nucleic acids closely related thereto as described herein.

to that of the probe can then be distinguished using stringent (also, "high stringency") hybridization conditions. By sequencing the individual clones thus identified with sequencing primers designed from the original sequence it is then possible to extend the sequence in both directions to determine the full length sequence. Suitable techniques are described, for example, in *Current Protocols in Molecular Biology* (F.M. Ausubel et al, eds), containing supplements through Supplement 42, 1998, John Wiley and Sons, Inc., especially chapters 5, 6 and 7.

Embodiments of the invention include isolated nucleic acid molecules comprising any of the following nucleotide sequences: 1.) a nucleotide sequence which encodes a protein comprising the amino acid sequence of hsFATP1 (SEQ ID

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NO:47), the amino acid sequence of hsFATP2 (SEQ ID NO:49), the amino acid sequence of hsFATP3 (SEQ ID NO:¹⁰⁰~~51~~), the amino acid sequence of hsFATP4 (SEQ ID NO: 53), the amino acid sequence of hsFATP5 (SEQ ID NO:55) or the amino acid sequence of hsFATP6 (SEQ ID NO:57); 2.) nucleotide sequences of hsFATP1, 5 hsFATP2, hsFATP3, hsFATP4, hsFATP5, or hsFATP6 (SEQ ID NO:46, 48, 101, 52, 54, or 56, respectively); 3.) a nucleotide sequence which is complementary to the nucleotide sequence of hsFATP1 (SEQ ID NO:46), hsFATP2 (SEQ ID NO:48), hsFATP3 (SEQ ID NO:101), hsFATP4 (SEQ ID NO:52), hsFATP5 (SEQ ID NO:54) or hsFATP6 (SEQ ID NO:56); 4.) a nucleotide sequence which consists of the coding 10 region of hsFATP1 (SEQ ID NO:46), the coding region of hsFATP2 (SEQ ID NO:48), the coding region of hsFATP3 (SEQ ID NO:101), the coding region of hsFATP4 (SEQ ID NO:52), the coding region of hsFATP5 (SEQ ID NO:54), or the coding region of hsFATP6 (SEQ ID NO:56).

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The invention further relates to nucleic acids (nucleic acid molecules or 15 polynucleotides) having nucleotide sequences identical over their entire length to those shown in the figures, for instance Figures 44A-44C (SEQ ID NO:46), Figures 46A and 46B (SEQ ID NO:48), Figures 94A and 94B (SEQ ID NO:101), Figures 50A-50C (SEQ ID NO:52), Figure 52 (SEQ ID NO:54), and Figures 54A-54C (SEQ ID NO:56). It further relates to DNA, which due to the degeneracy of the genetic code, encodes a 20 FATP encoded by one of the FATP-encoding DNAs, whose amino acid sequence is provided herein. Also provided by the invention are nucleic acids having the coding sequences for the mature polypeptides or fragments in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence. The nucleic acids of the invention encompass nucleic acids 25 that include a single continuous region or discontinuous regions encoding the polypeptide, together with additional regions, that may also contain coding or non-coding sequences. The nucleic acids may also contain non-coding sequences, including, for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences, termination signals, ribosome binding sites, sequences that

stabilize mRNA, introns, polyadenylation signals, and additional coding sequences which encode additional amino acids. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain embodiments of the invention, the marker sequence can be a hexa-histidine peptide, as provided in the pQE
5 vector (Qiagen, Inc.) and described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), or an HA tag (Wilson *et al.*, *Cell* 37: 767 (1984)), or a sequence encoding glutathione S-transferase of *Schistosoma japonicum* (vectors available from Pharmacia; see Smith, D.B. and Johnson K.S., *Gene* 67:31 (1988) and Kaelin, W.G. *et al.*, *Cell* 70:351 (1992)). Nucleic acids of the invention also include, but are not limited to,
10 nucleic acids comprising a structural gene and its naturally associated sequences that control gene expression.

The invention further relates to variants, including naturally-occurring allelic variants, of those nucleic acids described specifically herein by DNA sequence, that encode variants of such polypeptides as those having the amino acid sequences shown
15 in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:102), Figure 51 (SEQ ID NO:53) Figure 53 (SEQ ID NO:55), or Figure 55 (SEQ ID NO:57). Such variants include nucleic acids encoding variants of the above-listed amino acid sequences, wherein those variants have several, such as 5 to 10, 1 to 5, or 3, 2 or 1 amino acids substituted, deleted, or added, in any combination. Variants include
20 polynucleotides encoding polypeptides with at least 95% but less than 100% amino acid sequence identity to the polypeptides described herein by amino acid sequence. Variant polynucleotides hybridize, under low to high stringency conditions, to the alleles described herein by DNA sequence. In one embodiment, variants have silent substitutions, additions and deletions that do not alter the properties and activities of the
25 FATP. Allelic variants of the polynucleotides encoding hsFATP1 (Figure 45; SEQ ID NO:47), hsFATP2 (Figure 47; SEQ ID NO:49), hsFATP3 (Figures 94A and 94B; SEQ ID NO:102), hsFATP4 (Figure 51; SEQ ID NO:53), Figure 53 (SEQ ID NO:55) and hsFATP6 (Figure 55; SEQ ID NO:57) will be identified as mapping to chromosomal locations listed for the corresponding wild type genes in Table 2 in Example 1.

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Orthologous genes are gene loci in different species that are sufficiently similar to each other in their nucleotide sequences to suggest that they originated from a common ancestral gene. Orthologous genes arise when a lineage splits into two species, rather than when a gene is duplicated within a genome. Proteins that are orthologs are
5 encoded by genes of two different species, wherein the genes are said to be orthologous.

The invention further relates to polynucleotides encoding polypeptides which are orthologous to those polypeptides having a specific amino acid sequence described herein, such as the amino acid sequences shown in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:102), Figure 51 (SEQ ID
10 NO:53), Figure 53 (SEQ ID NO:55), or Figure 55 (SEQ ID NO:57). These polynucleotides, which can be called ortholog polynucleotides, encode orthologous polypeptides that can range in amino acid sequence identity to a reference amino acid sequence described herein, from about 65% to less than 100%, but preferably 70% to 80%, more preferably 80% to 90%, and still more preferably 90% to less than 100%.
15 Orthologous polypeptides can also be those polypeptides that range in amino acid sequence similarity to a reference amino acid sequence described herein from about 75% to 100%, within the signature sequence. The amino acid sequence similarity between the signature sequences of orthologous polypeptides is preferably 80%, more preferably 90%, and still more preferably, 95%. The ortholog polynucleotides encode
20 polypeptides that have similar functional characteristics (e.g., fatty acid transport activity) and similar tissue distribution, as appropriate to the organism from which the ortholog polynucleotides can be isolated.

Ortholog polynucleotides can be isolated from (e.g., by cloning or nucleic acid amplification methods) a great number of species, as shown by the sample of FATPs
25 from evolutionarily divergent species described herein (see, e.g., Figures 44A-C through Figure 89). Ortholog polynucleotides corresponding to those in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:101), Figure 51 (SEQ ID NO:53), Figure 52 (SEQ ID NO:54) and Figure 55 (SEQ ID NO:57) are

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those which can be isolated from mammals such as rat, dog, chimpanzee, monkey, baboon, pig, rabbit and guinea pig, for example.

Further variants that are fragments of the nucleic acids of the invention may be used to synthesize full-length nucleic acids of the invention, such as by use as primers in a polymerase chain reaction. As used herein, the term primer refers to a single-stranded oligonucleotide which acts as a point of initiation of template-directed DNA synthesis under appropriate conditions (e.g., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer, but typically ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template, but must be sufficiently complementary to hybridize with a template. The term primer site refers to the area of the target DNA to which a primer hybridizes. The term primer pair refers to a set of primers including a 5' (upstream) primer that hybridizes with the 5' end of the DNA sequence to be amplified and a 3' (downstream) primer that hybridizes with the complement of the 3' end of the sequence to be amplified.

Further embodiments of the invention are nucleic acids that are at least 80% identical over their entire length to a nucleic acid described herein, for example a nucleic acid having the nucleotide sequence in Figures 44A-44C (SEQ ID NO:46), Figures 46A-46B (SEQ ID NO:48), Figures 94A and 94B (SEQ ID NO:101), Figures 50A-50C (SEQ ID NO:52), Figure 52 (SEQ ID NO:54), and Figures 54A-54C (SEQ ID NO:56). Additional embodiments are nucleic acids, and the complements of such nucleic acids, having at least 90% nucleotide sequence identity to the above-described sequences, and nucleic acids having at least 95% nucleotide sequence identity. In preferred embodiments, DNA of the present invention has 97% nucleotide sequence identity, 98% nucleotide sequence identity, or at least 99% nucleotide sequence identity with the DNA whose sequences are presented herein.

Other embodiments of the invention are nucleic acids that are at least 80% identical in nucleotide sequence to a nucleic acid encoding a polypeptide having an amino acid sequence as set forth in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:102), Figure 51 (SEQ ID NO:53), Figure 53 (SEQ ID NO:55) or Figure 55 (SEQ ID NO:57), or as such amino acid sequences are set forth elsewhere herein, and nucleic acids that are complementary to such nucleic acids. Specific embodiments are nucleic acids having at least 90% nucleotide sequence identity to a nucleic acid encoding a polypeptide having an amino acid sequence as described in the list above, nucleic acids having at least 95% sequence identity, and nucleic acids having at least 97% sequence identity.

The terms "complementary" or "complementarity" as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. Complementarity between two single-stranded molecules may be "partial" in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single-stranded molecules (that is, when A-T and G-C base pairing is 100% complete). The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend on binding between nucleic acid strands.

The invention further includes nucleic acids that hybridize to the above-described nucleic acids, especially those nucleic acids that hybridize under stringent hybridization conditions. "Stringent hybridization conditions" or "high stringency conditions" generally occur within a range from about T_m minus 5°C (5° C below the strand dissociation temperature or melting temperature (T_m) of the probe nucleic acid molecule) to about 20° C to 25° C below T_m . As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect molecules having identical or related polynucleotide sequences. An example of high stringency hybridization follows. Hybridization solution is (6x SSC/10 mM EDTA/0.5% SDS/5x Denhardt's solution/100 µg/ml sheared and denatured salmon

sperm DNA). Hybridization is at 64-65°C for 16 hours. The hybridized blot is washed two times with 2x SSC/0.5% SDS solution at room temperature for 15 minutes each, and two times with 0.2x SSC/0.5% SDS at 65°C, for one hour each. Further examples of high stringency conditions can be found on pages 2.10.1-2.10.16 (see particularly
5 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., containing supplements up through Supplement 42, 1998). Examples of high, medium, and low stringency conditions can be found on pages 36 and 37 of WO 98/40404, which are incorporated herein by reference.

The invention further relates to nucleic acids obtainable by screening an
10 appropriate library with a probe having a nucleotide sequence such as that set forth in Figures 44A-44C (SEQ ID NO:46), Figures 46A-46B (SEQ ID NO:48), Figures 94A and 94B (SEQ ID NO:101), Figures 50A-50C (SEQ ID NO:52), Figure 52 (SEQ ID NO:54) or Figures 54A-54C (SEQ ID NO:56), or a probe which is a sufficiently long fragment of any of the above; and isolating the nucleic acid. Such probes generally can
15 comprise at least 15 nucleotides. Nucleic acids obtainable by such screenings may include RNAs, cDNAs and genomic DNA, for example, encoding FATPs of the FATP family described herein.

Further uses for the nucleic acid molecules of the invention, whether encoding a full-length FATP or whether comprising a contiguous portion of a nucleic acid molecule
20 such as one given in SEQ ID NO:46, 48, 101, 52, 54, or 56, include use as markers for tissues in which the corresponding protein is preferentially expressed (to identify constitutively expressed proteins or proteins produced at a particular stage of tissue differentiation or stage of development of a disease state); as molecular weight markers on southern gels; as chromosome markers or tags (when labeled, for example with
25 biotin, a radioactive label or a fluorescent label) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in a mammal to identify potential genetic disorders; as probes to hybridize and thus identify, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of

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discovering other novel nucleic acid molecules; for selecting and making oligomers for attachment to a "gene chip" or other support, to be used, for example, for examination of expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or to elicit another immune response.

Further methods to obtain nucleic acids encoding FATPs of the FATP family include PCR and variations thereof (e.g., "RACE" PCR and semi-specific PCR methods). Portions of the nucleic acids having a nucleotide sequence set forth in Figures 44A-44C (SEQ ID NO:46), Figures 46A-46B (SEQ ID NO:48), Figures 94A and 94B (SEQ ID NO:101), Figures 50A-50C (SEQ ID NO:52), Figure 52 (SEQ ID NO:54) or Figures 54A-54C (SEQ ID NO:56), (especially "flanking sequences" on either side of a coding region) can be used as primers in methods using the polymerase chain reaction, to produce DNA from an appropriate template nucleic acid.

Once a fragment of the FATP gene is generated by PCR, it can be sequenced, and the sequence of the product can be compared to other DNA sequences, for example, by using the BLAST Network Service at the National Center for Biotechnology Information. The boundaries of the open reading frame can then be identified using semi-specific PCR or other suitable methods such as library screening. Once the 5' initiator methionine codon and the 3' stop codon have been identified, a PCR product encoding the full-length gene can be generated using genomic DNA as a template, with primers complementary to the extreme 5' and 3' ends of the gene or to their flanking sequences. The full-length genes can then be cloned into expression vectors for the production of functional proteins.

The invention also relates to isolated proteins or polypeptides such as those encoded by nucleic acids of the present invention. Isolated proteins can be purified from a natural source or can be made recombinantly. Proteins or polypeptides referred to herein as "isolated" are proteins or polypeptides that exist in a state different from the state in which they exist in cells in which they are normally expressed in an organism, and include proteins or polypeptides obtained by methods described herein, similar

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deletion or substitution of one or more amino acid residues, or modified polypeptides in which one or more residues are modified, and mutants comprising one or more modified residues. Portions or fragments of a FATP can range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

5 The isolated proteins of the invention preferably include mammalian fatty acid transport proteins of the FATP family of homologous proteins. In one embodiment, the extent of amino acid sequence similarity between a polypeptide having one of the amino acid sequences shown in Figure 45 (SEQ ID NO:47), Figure 47 (SEQ ID NO:49), Figures 94A and 94B (SEQ ID NO:102), Figure 51 (SEQ ID NO:53), Figure 53 (SEQ
10 ID NO:55), or Figure 55 (SEQ ID NO:57), and the respective functional equivalents of these polypeptides is at least about 88%. In other embodiments, the degree of amino acid sequence similarity between a FATP and its respective functional equivalent is at least about 91%, at least about 94%, or at least about 97%.

 The polypeptides of the invention also include those FATPs encoded by
15 polynucleotides which are orthologous to those polynucleotides, the sequences of which are described herein in whole or in part. FATPs which are orthologs to those described herein by amino acid sequence, in whole or in part, are, for example fatty acid transport proteins 1-6 of dog, rat chimpanzee, monkey, rabbit, guinea pig, baboon and pig, and are also embodiments of the invention.

20 To determine the percent identity or similarity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment, and non-homologous (dissimilar) sequences can be disregarded for comparison purposes). In a preferred embodiment, the
25 length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first

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TABLE 1. Conservative Amino Acid Substitutions

Aromatic		Phenylalanine	
		Tryptophan	
		Tyrosine	
Hydrophobic		Leucine	
		Isoleucine	
		Valine	
Polar		Glutamine	
		Asparagine	
Basic		Arginine	
		Lysine	
		Histidine	
Acidic		Aspartic Acid	
		Glutamic Acid	
Small		Alanine	
		Serine	
		Threonine	
		Methionine	
		Glycine	

The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm.

- 10 (Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence

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Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereaux, J., eds., M. Stockton Press, New York, 1991). In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* 5 (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the 10 GCG software package (Devereux, J., *et al.*, *Nucleic Acids Res.* 12(1):387 (1984)) (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*CABIOS*, 4:11-17 (1989)) 15 which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against databases to, for example, identify other family members or related sequences. Such searches can be performed 20 using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (*J. Mol. Biol.* 215:403-10 (1990)). BLAST nucleotide searches can be performed with the NBLAST program, score = 100, word length = 12 to obtain nucleotide sequences homologous to (with calculatably significant similarity to) the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, 25 score = 50, word length = 3 to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (*Nucleic Acids Res.* 25(17):3389-3402 (1997)). When utilizing BLAST and gapped BLAST programs, the default

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parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

Similarity for nucleotide and amino acid sequences can be defined in terms of the parameters set by the Advanced Blast search available from NCBI (the National Center for Biotechnology Information; see, for Advanced BLAST page, www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=1). These default parameters, recommended for a query molecule of length greater than 85 amino acid residues or nucleotides have been set as follows: gap existence cost, 11, per residue gap cost, 1; lambda ratio, 0.85. Further explanation of version 2.0 of BLAST can be found on related website pages and in Altschul, S.F. *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997).

The invention further relates to fusion proteins, comprising a FATP or functional portion thereof (as described above) as a first moiety, linked to second moiety not occurring in the FATP as found in nature. Thus, the second moiety can be an amino acid, peptide or polypeptide. The first moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises a FATP as the first moiety, and a second moiety comprising a linker sequence and an affinity ligand. Fusion proteins can be produced by a variety of methods. For example, a fusion protein can be produced by the insertion of a FATP gene or portion thereof into a suitable expression vector, such as Bluescript SK +/- (Stratagene), pGEX-4T-2 (Pharmacia), pET-24(+) (Novagen), or vectors of similar construction. The resulting construct can be introduced into a suitable host cell for expression. Upon expression, fusion protein can be purified from cells by means of a suitable affinity matrix (See e.g., *Current Protocols in Molecular Biology*, Ausubel, F.M. *et al.*, eds., Vol. 2, pp. 16.4.1-16.7.8, containing supplements up through Supplement 42, 1998).

The invention also relates to enzymatically produced, synthetically produced, or recombinantly produced portions of a fatty acid transport protein. Portions of a FATP can be made which have full or partial function on their own, or which when mixed

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together (though fully, partially, or nonfunctional alone), spontaneously assemble with one or more other polypeptides to reconstitute a functional protein having at least one function characteristic of a FATP.

Fragments of a FATP can be produced by direct peptide synthesis, for example
5 those using solid-phase techniques (Roberge, J.Y. *et al.*, *Science* 269:202-204 (1995);
Merrifield, J., *J. Am. Chem. Soc.* 85:2149-2154 (1963)). Protein synthesis can be
performed using manual techniques or by automation. Automated synthesis can be
carried out using, for instance, an Applied Biosystems 431A Peptide Synthesizer
(Perkin Elmer). Various fragments of a FATP can be synthesized separately and
10 combined using chemical methods.

One aspect of the invention is a peptide or polypeptide having the amino acid
sequence of a portion of a fatty acid transport protein which is hydrophilic rather than
hydrophobic, and ordinarily can be detected as facing the outside of the cell membrane.
Such a peptide or polypeptide can be thought of as being an extracellular domain of the
15 FATP, or a mimetic of said extracellular domain. It is known, for example, that a
portion of human FATP4 that includes a highly conserved motif is involved in AMP-
CoA binding function (Stuhlsatz-Krouper, S.M. *et al.*, *J. Biol. Chem.* 44:28642-28650
(1998)).

The term "mimetic" as used herein, refers to a molecule, the structure of which
20 is developed from knowledge of the structure of the FATP of interest, or one or more
portions thereof, and, as such, is able to effect some or all of the functions of a FATP.

Portions of an FATP can be prepared by enzymatic cleavage of the isolated
protein, or can be made by chemical synthesis methods. Portions of a FATP can also be
made by recombinant DNA methods in which restriction fragments, or fragments that
25 may have undergone further enzymatic processing, or synthetically made DNAs are
joined together to construct an altered FATP gene. The gene can be made such that it
encodes one or more desired portions of a FATP. These portions of FATP can be
entirely homologous to a known FATP, or can be altered in amino acid sequence
relative to naturally occurring FATPs to enhance or introduce desired properties such as

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solubility, stability, or affinity to a ligand. A further feature of the gene can be a sequence encoding an N-terminal signal peptide directed to the plasma membrane.

An extracellular domain can be determined by a hydrophobicity plot, such as those shown in Figures 28A, 29A, and 35A, or by a hydrophilicity plot such as those
5 shown in Figures 28C, 29C, 35C, 91, 92 and 93. A polypeptide or peptide comprising all or a portion of a FATP extracellular domain can be used in a pharmaceutical composition. When administered to a mammal by an appropriate route, the polypeptide or peptide can bind to fatty acids and compete with the native FATPs in the membrane of cells, thereby making fewer fatty acid molecules available as substrates for transport
10 into cells, and reducing the amount of fatty acids taken up by, for example, the heart, in the case of FATP6.

Another aspect of the invention relates to a method of producing a fatty acid transport protein, variants or portions thereof, and to expression systems and host cells containing a vector appropriate for expression of a fatty acid transport protein.

15 Cells that express a FATP, a variant or a portion thereof, or an ortholog of a FATP described herein by amino acid sequence, can be made and maintained in culture, under conditions suitable for expression, to produce protein in the cells for cell-based assays, or to produce protein for isolation. These cells can be procaryotic or eucaryotic. Examples of procaryotic cells that can be used for expression include *Escherichia coli*,
20 *Bacillus subtilis* and other bacteria. Examples of eucaryotic cells that can be used for expression include yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and other lower eucaryotic cells, and cells of higher eucaryotes such as those from insects and mammals, such as primary cells and cell lines such as CHO, HeLa, 3T3 and BHK cells, preferably COS cells and human kidney 293 cells, and
25 more preferably Jurkat cells. (See, e.g., Ausubel, F.M. *et al.*, eds. *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, Inc., containing Supplements up through Supplement 42, 1998)).

In one embodiment, host cells that produce a recombinant FATP, or a portion thereof, a variant, or an ortholog of a FATP described herein by amino acid sequence,

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can be made as follows. A gene encoding a FATP, variant or a portion thereof can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, phage, cosmid, phagemid, virus, virus-derived vector (e.g., SV40, vaccinia, adenovirus, fowl pox virus, pseudorabies viruses, retroviruses) or other suitable replicon, which can be present in a single copy or multiple copies, or the gene can be integrated in a host cell chromosome. A suitable replicon or integrated gene can contain all or part of the coding sequence for a FATP or variant, operably linked to one or more expression control regions whereby the coding sequence is under the control of transcription signals and linked to appropriate translation signals to permit translation. The vector can be introduced into cells by a method appropriate to the type of host cells (e.g., transfection, electroporation, infection). For expression from the FATP gene, the host cells can be maintained under appropriate conditions (e.g., in the presence of inducer, normal growth conditions, etc.). Proteins or polypeptides thus produced can be recovered (e.g., from the cells, as in a membrane fraction, from the periplasmic space of bacteria, from culture medium) using suitable techniques. Appropriate membrane targeting signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention can be recovered and purified from cell cultures (or from their primary cell source) by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and high performance liquid chromatography. Known methods for refolding protein can be used to regenerate active conformation if the polypeptide is denatured during isolation or purification.

In a further aspect of the invention are methods for assessing the transport function of any of the fatty acid transport proteins or polypeptides described herein, including orthologs, and in variations of these, methods for identifying an inhibitor (or an enhancer) of such function and methods for assessing the transport function in the presence of a candidate inhibitor or a known inhibitor.

A variety of systems comprising living cells can be used for these methods. Cells to be used in fatty acid transport assays, and further in methods for identifying an inhibitor or enhancer of this function, express one or more FATPs. See Examples 3, 6, 9, 12 and 14 for data on tissue distribution of expression of FATPs, and Examples 10 and 11 describing recombinant cells expressing FATP. Cells for use in cell-based assays described herein can be drawn from a variety of sources, such as isolated primary cells of various organs and tissues wherein one or more FATPs are naturally expressed. In some cases, the cells can be from adult organs, and in some cases, from embryonic or fetal organs, such as heart, lung, liver, intestine, skeletal muscle, kidney and the like. Cells for this purpose can also include cells cultured as fragments of organs or in conditions simulating the cell type and/or tissue organization of organs, in which artificial materials may be used as substrates for cell growth. Other types of cells suitable for this purpose include cells of a cell strain or cell line (ordinarily comprising cells considered to be "transformed") transfected to express one or more FATPs.

A further embodiment of the invention is a method for detecting, in a sample of cells, a fatty acid transport protein, a portion or fragment thereof, a fusion protein comprising a FATP or a portion thereof, or an ortholog as described herein, wherein the cells can be, for instance, cells of a tissue, primary culture cells, or cells of a cell line, including cells into which nucleic acid has been introduced. The method comprises adding to the sample an agent that specifically binds to the protein, and detecting the agent specifically bound to the protein. Appropriate washing steps can be added to reduce nonspecific binding to the agent. The agent can be, for example, an antibody, a ligand or a substrate mimic. The agent can have incorporated into it, or have bound to it, covalently or by high affinity non-covalent interactions, for instance, a label that facilitates detection of the agent to which it is bound, wherein the label can be, but is not limited to, a phosphorescent label, a fluorescent label, a biotin or avidin label, or a radioactive label. The means of detection of a fatty acid transport protein can vary, as appropriate to the agent and label used. For example, for an antibody that binds to the fatty acid transport protein, the means of detection may call for binding a second

antibody, which has been conjugated to an enzyme, to the antibody which binds the fatty acid transport protein, and detecting the presence of the second antibody by means of the enzymatic activity of the conjugated enzyme.

Similar principles can also be applied to a cell lysate or a more purified
5 preparation of proteins from cells that may comprise a fatty acid transport protein of interest, for example in the methods of immunoprecipitation, immunoblotting, immunoaffinity methods, that in addition to detection of the particular FATP, can also be used in purification steps, and qualitative and quantitative immunoassays. See, for instance, chapters 11 through 14 in *Antibodies: A Laboratory Manual*, E. Harlow and
10 D. Lane, eds., Cold Spring Harbor Laboratory, 1988.

Isolated fatty acid transport protein or, an antigenically similar portion thereof, especially a portion that is soluble, can be used in a method to select and identify molecules which bind specifically to the FATP. Fusion proteins comprising all of, or a portion of, the fatty acid transport protein linked to a second moiety not occurring in the
15 FATP as found in nature, can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in which the second moiety comprises an affinity ligand (e.g., an enzyme, antigen, epitope). FATP fusion proteins can be produced by the insertion of a gene encoding the FATP or a variant thereof, or a suitable portion of such gene into a suitable expression vector, which encodes an
20 affinity ligand (e.g., pGEX-4T-2 and pET-15b, encoding glutathione S-transferase and His-Tag affinity ligands, respectively). The expression vector can be introduced into a suitable host cell for expression. Host cells are lysed and the lysate, containing fusion protein, can be bound to a suitable affinity matrix by contacting the lysate with an affinity matrix.

25 In one embodiment, the fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand portion of the fusion protein to the matrix, and is contacted with one or more candidate binding agents (e.g., a mixture of peptides) to be tested, under conditions suitable for binding of the binding agents to the FATP portion of the bound fusion protein. Next, the affinity matrix with

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bound fusion protein can be washed with a suitable wash buffer to remove unbound candidate binding agents and non-specifically bound candidate binding agents. Those agents which remain bound can be released by contacting the affinity matrix with fusion protein bound thereto with a suitable elution buffer. Wash buffer can be formulated to permit binding of the fusion protein to the affinity matrix, without significantly disrupting binding of specifically bound binding agents. In this aspect, elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the candidate binding agents to the target portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of specifically bound agent, or the elution buffer can comprise a release component or components designed to disrupt binding of specifically bound agent to the target portion of the fusion protein.

Immobilization can be performed prior to, simultaneous with, or after, contacting the fusion protein with candidate binding agent, as appropriate. Various permutations of the method are possible, depending upon factors such as the candidate molecules tested, the affinity matrix-ligand pair selected, and elution buffer formulation. For example, after the wash step, fusion protein with binding agent molecules bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer, such as glutathione for a GST fusion). Where the fusion protein comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with the candidate agent bound thereto. Bound agent molecules can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

One or more candidate binding agents can be tested simultaneously. Where a mixture of candidate binding agents is tested, those found to bind by the foregoing processes can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). Large libraries of candidate binding agents (e.g., peptides, RNA oligonucleotides) produced by combinatorial chemical synthesis or by other methods can be tested (see e.g., Ohlmeyer, M.H.J. *et al.*, *Proc. Natl. Acad. Sci.*

USA 90:10922-10926 (1993) and DeWitt, S.H. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6909-6913 (1993), relating to tagged compounds; see also Rutter, W.J. *et al.* U.S. Patent No. 5,010,175; Huebner, V.D. *et al.*, U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Random sequence RNA libraries (see Ellington, A.D. *et al.*, *Nature* 346:818-822 (1990); Bock, L.C. *et al.*, *Nature* 355:584-566 (1992); and Szostak, J.W., *Trends in Biochem. Sci.* 17:89-93 (March, 1992)) can also be screened according to the present method to select RNA molecules which bind to a target FATP or FATP fusion protein. Where binding agents selected from a combinatorial library by the present method carry unique tags, identification of individual biomolecules by chromatographic methods is possible. Where binding agents do not carry tags, chromatographic separation, followed by mass spectrometry to ascertain structure, can be used to identify binding agents selected by the method, for example.

The invention also comprises a method for identifying an agent which inhibits interaction between a fatty acid transport protein (e.g., one comprising the amino acid sequence in SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:102, or SEQ ID NO:57), and a ligand of said protein. The FATP can be one described by amino acid sequence herein, a portion or fragment thereof, a variant thereof, or an ortholog thereof, or a FATP fusion protein. Here, a ligand can be, for instance, a substrate, or a substrate mimic, an antibody, or a compound, such as a peptide, that binds with specificity to a site on the protein. The method comprises combining, not limited to a particular order, the fatty acid protein, the ligand of the protein, and a candidate agent to be assessed for its ability to inhibit interaction between the protein and the ligand, under conditions appropriate for interaction between the protein and the ligand (e.g., pH, salt, temperature conditions conducive to appropriate conformation and molecular interactions); determining the extent to which the protein and ligand interact; and comparing (1) the extent of protein-ligand interaction in the presence of candidate agent with (2) the extent of protein-ligand interaction in the

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absence of candidate agent, wherein if (1) is less than (2), then the candidate agent is one which inhibits interaction between the protein and the ligand.

- The method can be facilitated, for example, by using an experimental system which employs a solid support (column chromatography matrix, wall of a plate, microtiter wells, column pore glass, pins to be submerged in a solution, beads, etc.) to which the protein can be attached. Accordingly, in one embodiment, the protein can be fixed to a solid phase directly or indirectly, by a linker. The candidate agent to be tested is added under conditions conducive for interaction and binding to the protein. The ligand is added to the solid phase system under conditions appropriate for binding.
- Excess ligand is removed, as by a series of washes done under conditions that do not disrupt protein-ligand interactions. Detection of bound ligand can be facilitated by using a ligand that carries a label (e.g., fluorescent, chemiluminescent, radioactive). In a control experiment, protein and ligand are allowed to interact in the absence of any candidate agent, under conditions otherwise identical to those used for the "test" conditions where candidate inhibiting agent is present, and any washes used in the test conditions are also used in the control. The extent to which ligand binds to the protein in the presence of candidate agent is compared to the extent to which ligand binds to the protein in the absence of the candidate agent. If the extent to which interaction of the protein and the ligand occurs is less in the presence of the candidate agent than in the absence of the candidate agent, the candidate agent is an agent which inhibits interaction between the protein and the ligand of the protein.

- In a further embodiment, an inhibitor (or an enhancer) of a fatty acid transport protein can be identified. The method comprises steps which are, or are variations of the following: contacting the cells with fatty acid, wherein the fatty acid can be labeled for convenience of detection; contacting a first aliquot of the cells with an agent being tested as an inhibitor (or enhancer) of fatty acid uptake while maintaining a second aliquot of cells under the same conditions but without contact with the agent; and measuring (e.g., quantitating) fatty acid in the first and second aliquots of cells; wherein a lesser quantity of fatty acid in the first aliquot compared to that in the second aliquot

is indicative that the agent is an inhibitor of fatty acid uptake by a fatty acid transport protein. A greater quantity of fatty acid in the first aliquot compared to that in the second aliquot is indicative that the agent is an enhancer of fatty acid uptake by a fatty acid transport protein.

5 A particular embodiment of identifying an inhibitor or enhancer of fatty acid transport function employs the above steps, but also employs additional steps preceding those given above: introducing into cells of a cell strain or cell line ("host cells" for the intended introduction of, or after the introduction of, a vector) a vector comprising a fatty acid transport protein gene, wherein expression of the gene can be regulatable or
10 constitutive, and providing conditions to the host cells under which expression of the gene can occur.

 The terms "contacting" and "combining" as used herein in the context of bringing molecules into close proximity to each other, can be accomplished by conventional means. For example, when referring to molecules that are soluble,
15 contacting is achieved by adding the molecules together in a solution. "Contacting" can also be adding an agent to a test system, such as a vessel containing cells in tissue culture.

 The term "inhibitor" or "antagonist", as used herein, refers to an agent which blocks, diminishes, inhibits, hinders, limits, decreases, reduces, restricts or interferes
20 with fatty acid transport into the cytoplasm of a cell, or alternatively and additionally, prevents or impedes the cellular effects associated with fatty acid transport. The term "enhancer" or "agonist", as used herein, refers to an agent which augments, enhances, or increases fatty acid transport into the cytoplasm of a cell. An antagonist will decrease fatty acid concentration, fatty acid metabolism and byproduct levels in the cell, leading
25 to phenotypic and molecular changes.

 In order to produce a "host cell" type suitable for fatty acid uptake assays and for assays derived therefrom for identifying inhibitors or enhancers thereof, a nucleic acid vector can be constructed to comprise a gene encoding a fatty acid transport protein, for example, human FATP1, FATP2, FATP3, FATP4, FATP5, FATP6, a mutant or variant

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thereof, an ortholog of the human proteins, such as mouse orthologs or orthologs found in other mammals, or a FATP family protein of origin in an organism other than a mammal. The gene of the vector can be regulatable, such as by the placement of the gene under the control of an inducible or repressible promoter in the vector (e.g.,

5 inducible or repressible by a change in growth conditions of the host cell harboring the vector, such as addition of inducer, binding or functional removal of repressor from the cell milieu, or change in temperature) such that expression of the FATP gene can be turned on or initiated by causing a change in growth conditions, thereby causing the protein encoded by the gene to be produced, in host cells comprising the vector, as a

10 plasma membrane protein. Alternatively, the FATP gene can be constitutively expressed.

A vector comprising an FATP gene, such as a vector described herein, can be introduced into host cells by a means appropriate to the vector and to the host cell type. For example, commonly used methods such as electroporation, transfection, for

15 instance, transfection using CaCl_2 , and transduction (as for a virus or bacteriophage) can be used. Host cells can be, for example, mammalian cells such as primary culture cells or cells of cell lines such as COS cells, 293 cells or Jurkat cells. Host cells can also be, in some cases, cells derived from insects, cells of insect cell lines, bacterial cells, such as *E. coli*, or yeast cells, such as *S. cerevisiae*. It is preferred that the fatty acid transport

20 protein whose function is to be assessed, with or without a candidate inhibitor or enhancer, be produced in host cells whose ancestor cells originated in a species related to the species of origin of the FATP gene encoding the fatty acid transport protein. For example, it is preferable that tests of function or of inhibition or enhancement of a mammalian FATP be carried out in host mammalian cells producing the FATP, rather

25 than bacterial cells or yeast cells.

Host cells comprising a vector comprising a regulatable FATP gene can be treated so as to allow expression of the FATP gene and production of the encoded protein (e.g., by contacting the cells with an inducer compound that effects transcription from an inducible promoter operably linked to the FATP gene).

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The test agent (e.g., an agonist or antagonist) is added to the cells to be used in a fatty acid transport assay, in the presence or absence of test agent, under conditions suitable for production and/or maintenance of the expressed FATP in a conformation appropriate for association of the FATP with test agent and substrate. For example, 5 conditions under which an agent is assessed, such as media and temperature requirements, can, initially, be similar to those necessary for transport of typical fatty acid substrates across the plasma membrane. One of ordinary skill in the art will know how to vary experimental conditions depending upon the biochemical nature of the test agent. The test agent can be added to the cells in the presence of fatty acid, or in the 10 absence of fatty acid substrate, with the fatty acid substrate being added following the addition of the test agent. The concentration at which the test agent can be evaluated can be varied, as appropriate, to test for an increased effect with increasing concentrations.

Test agents to be assessed for their effects on fatty acid transport can be any 15 chemical (element, molecule, compound), made synthetically, made by recombinant techniques or isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules, such as antisense nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and compiled into 20 libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, esters, aldehydes, ethers and other classes of organic compounds. Test agents can also be natural or genetically engineered products isolated from lysates of cells, bacterial, animal or plant, or can be the cell lysates themselves. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of 25 compounds, especially in initial screening steps.

Thus, the invention relates to a method for identifying agents which alter fatty acid transport, the method comprising providing the test agent to the cell (wherein "cell" includes the plural, and can include cells of a cell strain, cell line or culture of primary cells or organ culture, for example), under conditions suitable for binding to its target,

whether to the FATP itself or to another target on or in the cell, wherein the transformed cell comprises a FATP.

- In greater detail, to test one or more agents or compounds (e.g., a mixture of compounds can conveniently be screened initially) for inhibition of the transport
- 5 function of a fatty acid transport protein, the agent(s) can be contacted with the cells. The cells can be contacted with a labeled fatty acid. The fatty acid can be, for example, a known substrate of the fatty acid transport protein such as oleate or palmitate. The fatty acid can itself be labeled with a radioactive isotope, (e.g., ^3H or ^{14}C) or can have a radioactively labeled adduct attached. In other variations, the fatty acid can have
- 10 chemically attached to it a fluorescent label, or a substrate for an enzyme occurring within the cells, wherein the substrate yields a detectable product, such as a highly colored or fluorescent product. Addition of candidate inhibitors and labeled substrate to the cells comprising fatty acid transport protein can be in either order or can be simultaneous.
- 15 A second aliquot of cells, which can be called "control" cells (a "first" aliquot of cells can be called "test" cells), is treated, if necessary (as in the case of transformed "host" cells), so as to allow expression of the FATP gene, and is contacted with the labeled substrate of the fatty acid transport protein. The second aliquot of cells is not contacted with one or more agents to be tested for inhibition of the transport function of
- 20 the protein produced in the cells, but is otherwise kept under the same culture conditions as the first aliquot of cells.

- In a further step of a method to identify inhibitors of a fatty acid transport protein, the labeled fatty acid is measured in the first and second aliquots of cells. A preliminary step of this measurement process can be to separate the external medium
- 25 from the cells so as to be able to distinguish the labeled fatty acid external to the cells from that which has been transported inside the cells. This can be accomplished, for instance, by removing the cells from their growth container, centrifuging the cell suspension, removing the supernatant and performing one or more wash steps to extensively dilute the remaining medium which may contain labeled fatty acid.

Detection of the labeled fatty acid can be by a means appropriate to the label used. For example, for a radioactive label, detection can be by scintillation counting of appropriately prepared samples of cells (e.g., lysates or protein extracts); for a fluorescent label, by measuring fluorescence in the cells by appropriate instrumentation.

5 If a compound tested as a candidate inhibitor of transport function causes the test cells to have less labeled fatty acid detected in the cells than that detected in the control cells, then the compound is an inhibitor of the fatty acid transport protein. Procedures analogous to those above can be devised for identifying enhancers (agonists of FATPs) of fatty acid transport function wherein if the test cells contain more labeled fatty acid
10 than that detected in the control cells, or if the fatty acid is taken up at a higher rate, then the compound being tested can be concluded to be an enhancer of the fatty acid transport protein.

Example 13 describes use of an assay of this type to identify an inhibitor of a FATP. In Example 13, an antisense oligonucleotide which specifically inhibits
15 biosynthesis of mmFATP4 was demonstrated to inhibit fatty acid uptake into mouse enterocytes. Similarly, antisense oligonucleotides directed towards specifically inhibiting the biosynthesis of FATP6 in heart cells, FATP5 in liver cells, FATP3 in lung cells, and FATP2 in colon cells, can be demonstrated as examples of "test agents" that inhibit fatty acid transport.

20 Another assay to determine whether an agent is an inhibitor (or enhancer) of fatty acid transport employs animals, one or more of which are administered the agent, and one or more of which are maintained under similar conditions, but are not administered the agent. Both groups of animals are given fatty acids (e.g., orally, intravenously, by tube inserted into stomach or intestine), and the fatty acids taken up
25 into a bodily fluid (e.g., serum) or into an organ or tissue of interest are measured from comparable samples taken from each group of animals. The fatty acids may carry a label (e.g., radioactive) to facilitate detection and quantitation of fatty acids taken up into the fluid or tissue being sampled. This type of assay can be used alone or can be used in addition to *in vitro* assays of a candidate inhibitor or enhancer.

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An agent determined to be an inhibitor (or enhancer) of FATP function, such as fatty acid binding and/or fatty acid uptake, can be administered to cells in culture, or *in vivo*, to a mammal (e.g. human) to inhibit (or enhance) FATP function. Such an agent may be one that acts directly on the FATP (for example, by binding) or can act on an intermediate in a biosynthetic pathway to produce FATP, such as transcription of the FATP gene, processing of the mRNA, or translation of the mRNA. An example of such an agent is antisense oligonucleotide.

Antisense methods similar to those illustrated in Example 13 can be used to determine the target FATP of a compound or agent that has an inhibitory or enhancing effect on fatty acid uptake. For example, antisense oligonucleotide directed to the inhibition of FATP4 biosynthesis can be added to lung cells or cell lines derived from lung cells. In addition, antisense oligonucleotides directed to the inhibition of other FATPs, except for FATP3, can also be added to the lung cells. The administration of antisense oligonucleotides in this manner ensures that the predominant FATP activity remaining in the cells comes from FATP3. After a period of incubation of the cells with the antisense oligonucleotides sufficient to deplete the plasma membrane of the FATPs whose biosynthesis has been inhibited, a test agent, preferably one that has been shown by some preliminary test to have an inhibitory or enhancing activity on fatty acid transport, can be added to the lung cells. If the test agent is now demonstrated, after treatment of the cells with antisense oligonucleotides, to have an inhibitory or enhancing activity on fatty acid transport in the lung cells, it can be concluded that the target of the test agent is FATP3, or a molecule involved in the biosynthesis or activity of FATP3.

In another type of cell-based assay for uptake of fatty acids, a change of intracellular pH resulting from the uptake of fatty acids can be followed by an indicator fluorophore. The fluorophore can be taken up by the cells in a preincubation step. Fatty acids can be added to the cell medium, and after some period of incubation to allow FATP-mediated uptake of fatty acids, the change in λ_{\max} of fluorescence can be measured, as an indicator of a change in intracellular pH, as the λ_{\max} of fluorescence of

the fluorophore changes with the pH of its environment, thereby indicating uptake of fatty acids. One such fluorophore is BCECF (2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; Rink, T.J. *et al.*, *J. Cell. Biol.* 95: 189 (1982)).

In assays similar to those described above, a candidate inhibitor or enhancer of fatty acid transport function can be added (or mock-added, for control cultures) to cultures of cells engineered to express a desired FATP to which fatty acid substrate is also added. Inhibition of fatty acid uptake is indicated by a lack of the drop in pH, indicating fatty acid uptake, that is seen in control cells. Enhancement of fatty acid uptake is indicated by a decrease in intracellular pH, as compared to control cells not receiving the candidate enhancer of fatty acid transport function.

Yeast cells can be used in a similar cell-based assay for the uptake of fatty acids mediated by a FATP, and such an assay can be adapted to a screening assay for the identification of agents that inhibit or enhance fatty acid uptake by an FATP. Yeast cells lacking an endogenous FATP activity (mutated, disrupted or deleted for *FAT1*; Faergeman, N.J. *et al.*, *J. Biol. Chem.* 272(13):8531-8538 (1997); Watkins, P.A. *et al.*, *J. Biol. Chem.* 273(29):18210-18219 (1998)) can be engineered to harbor a related gene of the family of FATP-encoding genes, such as a mammalian FATP (e.g., human FATP4).

Examples of expression vectors include pEG (Mitchell, D.A., *et al.*, *Yeast* 9:715-723 (1993)) and pDAD1 and pDAD2, which contain a *GALI* promoter (Davis, L. I. and Fink, G. R., *Cell* 61:965-978 (1990)). A variety of promoters are suitable for expression. Available yeast vectors offer a choice of promoters. In one embodiment, the inducible *GALI* promoter is used. In another embodiment, the constitutive *ADHI* promoter (alcohol dehydrogenase; Bennetzen, J. L. and Hall, B. D., *J. Biol. Chem.* 257:3026-3031 (1982)) can be used to express an inserted gene on glucose-containing media. An example of a vector suitable for expression of a heterologous FATP gene in yeast is pQB169.

With the introduced FATP gene providing the only fatty acid transport protein function for the yeast cells, it is possible to study effect of the heterologous FATP on

fatty acid transport into the yeast cells in isolation. Assays for the uptake of fatty acids into the yeast cells can be devised that are similar to those described above and/or those assays that have been illustrated in the Examples. Tests for candidate inhibitors or enhancers of the heterologous FATP can be done in cultures of yeast cells, wherein the yeast cells are incubated with fatty acid substrate and an agent to be tested as an inhibitor or enhancer of FATP function. FATP uptake after a period of time can be measured by analyzing the contents of the yeast cells for fatty acid substrate, as compared with control yeast cells incubated with the fatty acid, but not with the test agent. Yeast cells have the additional advantage, over mammalian cells in culture, for example, that yeast cells can be forced to rely upon fatty acids as their only source of carbon, if the growth medium supplied to the yeast cells is formulated to contain no other source of carbon. Thus, the effect of the heterologous FATP on fatty acid uptake and metabolism in the engineered yeast cells can be amplified. An agent that efficiently blocks transport function of the heterologous FATP could result in death of the yeast cells. Thus, in this case, inhibition of function of the heterologous FATP can result in loss of viability. A simple measure of viability is turbidity of the yeast suspension culture, which can be adapted to a high throughput screening assay for effects of various agents to be tested, using microtiter plates or similar devices for small-volume cultures of the engineered yeast cells.

Cell-free assays can also be used to measure the transport of fatty acids across a membrane, and therefor also to assess a test treatment or test agent for its effect on the rate or extent of fatty acid transport. An isolated FATP, for example in the presence of a detergent that preserves the native 3-dimensional structure of the FATP, or partially purified FATP, can be used in an artificial membrane system typically used to preserve the native conformation and activity of membrane proteins. Such systems include liposomes, artificial bilayers of phospholipids, isolated plasma membrane such as cell membrane fragments, cell membrane fractions, or cell membrane vesicles, and other systems in which the FATP can be properly oriented within the membrane to have transport activity. Assays for transport activity can be performed using methods

analogous to those that can be used in cells engineered to predominantly express one FATP whose function is to be measured. A labeled (e.g., radioactively labeled) fatty acid substrate can be incubated with one side of a bilayer or in a suspension of liposomes constructed to integrate a properly oriented FATP. The accumulation of fatty acids with time can be measured, using appropriate means to detect the label (e.g., scintillation counting of medium on each side of the bilayer, or of the contents of liposomes isolated from the surrounding medium). Assays such as these can be adapted to use for the testing of agents which might interact with the FATP to produce an inhibitory or an enhancing effect on the rate or extent of fatty acid transport. That is, the above-described assay can be done in the presence or absence of the agent to be tested, and the results compared.

For examples of isolation of membrane proteins (ADP/ATP carrier and uncoupling protein), reconstitution into phospholipid vesicles, and assays of transport, see Klingenberg, M. *et al.*, *Methods Enzymol.* 260:369-389 (1995). For an example of a membrane protein (phosphate carrier of *Saccharomyces cerevisiae*) that was purified and solubilized from *E. coli* inclusion bodies, see Schroer, A. *et al.*, *J. Biol. Chem.* 273: 14269-14276 (1998). The Glut1 glucose transporter of rat has been expressed in yeast. A crude membrane fraction of the yeast was prepared and reconstituted with soybean phospholipids into liposomes. Glucose transport activity could be measured in the liposomes (Kasahara, T. and Kasahara, M., *J. Biol. Chem.* 273: 29113-29117 (1998)). Similar methods can be applied to the proteins and polypeptides of the invention.

Another embodiment of the invention is a method for inhibiting fatty acid uptake in a mammal (e.g., a human), comprising administering to the mammal a therapeutically effective amount of an inhibitor of the transport function of one or more of the fatty acid transport proteins, thereby decreasing fatty acid uptake by cells comprising the fatty acid protein(s). Where it is desirable to reduce the uptake of fatty acids, for example, in the treatment of chronic obesity or as a part of a program of weight control or hyperlipidemia control in a human, one or more inhibitors of one or more of the fatty acid transport proteins can be administered in an effective dose, and by

an effective route, for example, orally, or by an indwelling device that can deliver doses to the small intestine. The inhibitor can be one identified by methods described herein, or can be one that is, for instance, structurally related to an inhibitor identified by methods described herein (e.g., having chemical adducts to better stabilize or solubilize the inhibitor). The invention further relates to compositions comprising inhibitors of fatty acid uptake in a mammal, which may further comprise pharmaceutical carriers suitable for administration to a subject mammal, such as sterile solubilizing or emulsifying agents.

A further embodiment of the present invention is a method of enhancing or increasing fatty acid uptake, such as enhancing or increasing LCFA uptake in the small intestine (e.g., to treat or prevent a malabsorption syndrome or other wasting condition) or in the liver (e.g., by an enhancer of FATP5 transport activity to treat acute liver failure) or in the kidney (e.g., by an enhancer of FATP2 transport activity to treat kidney failure). In this embodiment, a therapeutically effective amount of an enhancer of the transport function of one or more of the fatty acid transport proteins can be administered to a mammalian subject, with the result that fatty acid uptake in the small intestine is enhanced. In this embodiment, one or more enhancers of one or more of fatty acid transport proteins is administered in an effective dose and by a route (e.g., orally or by a device, such as an indwelling catheter or other device) which can deliver doses to the gut. The enhancer of FATP function (e.g., an enhancer of FATP4 function) can be identified by methods described herein or can be one that is structurally similar to an enhancer identified by methods described herein.

Aerobic reperfusion of ischemic myocardium is a common clinical event which can occur during such treatments as cardiac surgery, angioplasty, and thrombolytic therapy after a myocardial infarction. During reperfusion, a rapid recovery of myocardial energy production is essential for the complete recovery of contractile function. Not only the extent of recovery of myocardial energy metabolism but also the type of energy substrate used by the heart during reperfusion are important determinants of functional recovery. Circulating fatty acid levels increase following acute

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The antibodies of the present invention can be polyclonal or monoclonal. The term antibody is intended to encompass both polyclonal and monoclonal antibodies. Antibodies of the present invention can be raised against an appropriate immunogen, including proteins or polypeptides of the present invention, such as an isolated or
5 recombinant FATP1, FATP2, FATP3, FATP4, FATP5, FATP6, mtFATP, ceFATPa, ceFATPb, scFATP or portions thereof, or synthetic molecules, such as synthetic peptides (e.g., conjugated to a suitable carrier). Preferred embodiments are antibodies that bind to any of the following: hsFATP1, hsFATP2, hsFATP3, hsFATP4, hsFATP5 or hsFATP6. The immunogen can be a polypeptide comprising a portion of a FATP
10 and having at least one function of a fatty acid transport protein, as described herein.

The term antibody is also intended to encompass single chain antibodies, chimeric, humanized or primatized (CDR-grafted) antibodies and the like, as well as chimeric or CDR-grafted single chain antibodies, comprising portions from more than one species. For example, the chimeric antibodies can comprise portions of proteins
15 derived from two different species, joined together chemically by conventional techniques or prepared as a single contiguous protein using genetic engineering techniques (e.g., DNA encoding the protein portions of the chimeric antibody can be expressed to produce a contiguous protein chain. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S.
20 Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, U.S. Patent No. 5,585,089; and Queen *et al.*, European Patent No. EP 0 451 216 B1. See also, Newman, R. *et al.*, *BioTechnology*, 10:1455-1460 (1992), regarding
25 primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*, 242:423-426 (1988) regarding single chain antibodies.)

Whole antibodies and biologically functional fragments thereof are also encompassed by the term antibody. Biologically functional antibody fragments which can be used include those fragments sufficient for binding of the antibody fragment to a

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FATP to occur, such as Fv, Fab, Fab' and F(ab')₂ fragments. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or
5 more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

Preparation of immunizing antigen (whole cells comprising FATP on the cell surface or purified FATP), and polyclonal and monoclonal antibody production can be
10 performed using any suitable technique. A variety of methods have been described (See e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); Chapter 11 In *Current Protocols*
15 *In Molecular Biology*, Vol. 2 (containing supplements up through Supplement 42, 1998), Ausubel, F.M. *et al.*, eds., (John Wiley & Sons: New York, NY)). Generally, a hybridoma can be produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals
20 immunized with the antigen of interest. Immunization of animals can be by introduction of whole cells comprising fatty acid transport protein on the cell surface. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

25 Other suitable methods of producing or isolating antibodies (including human antibodies) of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., Hoogenboom *et al.*, WO 93/06213; Hoogenboom *et al.*, U.S. Patent No. 5,565,332; WO 94/13804, published June 23, 1994; and Dower, W.J. *et al.*, U.S. Patent No. 5,427,908), or which rely upon

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immunization of transgenic animals (e.g., mice) capable of producing a full repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,569,825; Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; and Kucherlapati, R. *et al.*, European Patent No. EP 0 463 151 B1).

Another aspect of the invention is a method for directing an agent to cardiac muscle. The differential expression of FATP6 in cardiac muscle but not in other tissue types allows for the specific targeting of drugs, diagnostic agents, tagging labels, histological stains or other substances specifically to cardiac muscle. A targeting vehicle can be used for the delivery of such a substance. Targeting vehicles which bind specifically to FATP6 can be linked to a substance to be delivered to the cells of cardiac muscle. The linkage can be, for instance, via one or more covalent bonds, or by high affinity non-covalent bonds. A targeting vehicle can be an antibody, for instance, or other compound (e.g., a fatty acid or fatty acid analog) which binds to FATP6 with high specificity.

Targeting vehicles specific to the heart-specific protein FATP6 have *in vivo* (e.g., therapeutic and diagnostic) applications. For example, an antibody which specifically binds to FATP6 can be conjugated to a drug to be targeted to the heart (e.g., a cardiac glycoside to treat congestive heart failure, or β -adrenergic agents, sodium channel blockers or calcium channel blockers to treat arrhythmias). A substance (e.g., a radioactive substance) which can be detected (e.g., a label) *in vivo* can also be linked to a targeting vehicle which specifically binds to a heart-specific protein such as FATP6, and the conjugate can be used as a labeling agent to identify cardiac muscle cells.

Targeting vehicles specific to FATP6 find further applications *in vitro*. For example, an FATP6-specific targeting vehicle, such as an antibody (a polyclonal preparation or monoclonal) which specifically binds to FATP6, can be linked to a substance which can be used as a stain for a tissue sample (e.g., horseradish peroxidase) to provide a method for the identification of cardiac muscle in a sample, as can be used in embryology studies, for example.

In a similar manner, an agent can be directed to the liver of a mammal, as FATP5 is expressed in liver but not in other tissue types. A targeting vehicle which specifically binds to FATP5 can be conjugated to a drug for delivery of the drug to the liver, such as a drug to treat hepatitis, Wilson's disease, lipid storage diseases and liver cancer. As with targeting vehicles specific to FATP6, targeting vehicles specific to FATP5 can be used in studying tissue samples *in vitro*.

The invention also relates to compositions comprising a modulator of FATP function. The term "modulate" as used herein refers to the ability of a molecule to alter the function of another molecule. Thus, modulate could mean, for example, inhibit, antagonize, agonize, upregulate, downregulate, induce, or suppress. A modulator has the capability of altering function of its target. Such alteration can be accomplished at any stage of the transcription, translation, expression or function of the protein, so that, for example, modulation of a target gene can be accomplished by modulation of the DNA or RNA encoding the protein, and the protein itself.

Antagonists or agonists (inhibitors or enhancers) of the FATPs of the invention, antibodies that bind a FATP, or mimetics of a FATP can be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a mammalian subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of an inhibitor or enhancer compound to be identified by an assay of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, ethanol, surfactants, such as glycerol, excipients such as lactose and combinations thereof. The formulation can be chosen by one of ordinary skill in the art to suit the mode of administration. The chosen route of administration will be influenced by the predominant tissue or organ location of the FATP whose function is to be inhibited or enhanced. For example, for affecting the function of FATP4, a preferred administration can be oral or through a tube inserted into the stomach (e.g., direct stomach tube or nasopharyngeal tube), or through other means to accomplish delivery to the small

intestine. The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

Compounds of the invention which are FATPs, FATP fusion proteins, FATP
5 mimetics, FATP gene-specific antisense poly- or oligonucleotides, inhibitors or
enhancers of a FATP may be employed alone or in conjunction with other compounds,
such as therapeutic compounds. The pharmaceutical compositions may be administered
in any effective, convenient manner, including administration by topical, oral, anal,
vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal,
10 transdermal or intradermal routes, among others. In therapy or as a prophylactic, the
active agent may be administered to an individual as an injectable composition, for
example as a sterile aqueous dispersion, preferably isotonic.

Alternatively, the composition may be formulated for topical application, for
example, in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops,
15 mouthwash, impregnated dressings and sutures and aerosols, and may contain
appropriate conventional additives, including, for example, preservatives, solvents to
assist drug penetration, and emollients in ointments and creams. Such topical
formulations may also contain compatible conventional carriers, for example cream or
ointment bases, and ethanol or oleyl alcohol for lotions.

20 In addition, the amount of the compound will vary depending on the size, age,
body weight, general health, sex, and diet of the host, and the time of administration, the
biological half-life of the compound, and the particular characteristics and symptoms of
the disorder to be treated. Adjustment and manipulation of established dose ranges are
well within the ability of those of skill in the art.

25 A further aspect of the invention is a method to identify a polymorphism, or the
presence of an alternative or variant allele of a gene in the genome of an organism (of
interest here, genes encoding FATPs). As used herein, polymorphism refers to the
occurrence of two or more genetically determined alternative sequences or alleles in a
population. A polymorphic locus may be as small as a base pair. Polymorphic markers

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include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The first identified allelic form, or the most frequently occurring form can be
5 arbitrarily designated as the reference (usually, "wildtype") form, and other allelic forms are designated as alternative (sometimes, "mutant" or "variant"). Diploid organisms may be homozygous or heterozygous for allelic forms.

An "allele" or "allelic sequence" is an alternative form of a gene which may result from at least one mutation in the nucleotide sequence. Alleles may result in
10 altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many allelic forms (polymorphism). Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

15 Several different types of polymorphisms have been reported. A restriction fragment length polymorphism (RFLP) is a variation in DNA sequence that alters the length of a restriction fragment (Botstein *et al.*, *Am. J. Hum. Genet.* 32:314-331 (1980)). The restriction fragment length polymorphism may create or delete a restriction site, thus changing the length of the restriction fragment. RFLPs have been widely used in
20 human and animal genetic analyses (see WO 90/13668; WO 90/11369; Donis-Keller, *Cell* 51:319-337 (1987); Lander *et al.*, *Genetics* 121:85-99 (1989)). When a heritable trait can be linked to a particular RFLP, the presence of the RFLP in an individual can be used to predict the likelihood that the individual will also exhibit the trait.

Other polymorphisms take the form of short tandem repeats (STRs) that include
25 tandem di-, tri- and tetra-nucleotide repeated motifs. These tandem repeats are also referred to as variable number tandem repeat (VNTR) polymorphisms. VNTRs have been used in identity and paternity analysis (US 5,075,217; Armour *et al.*, *FEBS Lett.* 307:113-115 (1992); Horn *et al.*, WO 91/14003; Jeffreys, EP 370,719), and in a large number of genetic mapping studies.

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human FATP gene, comprising preparing amplified, purified FATP DNA from a reference human and amplified, purified, FATP DNA from a "test" human to be

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Detection of Binding by Protein That Binds to Mismatches. Amplified DNA comprising the FATP gene or portion of the gene of interest from genomic DNA, for example, of a normal individual is prepared, using primers designed on the basis of the DNA sequences provided herein. Amplified DNA is also prepared, in a similar manner,
5 from genomic DNA of an individual to be tested for bearing a distinguishable allele. The primers used in PCR carry different labels, for example, primer 1 with biotin, and primer 2 with ^{32}P . Unused primers are separated from the PCR products, and the products are quantitated. The heteroduplexes are used in a mismatch detection assay using immobilized mismatch binding protein (MutS) bound to nitrocellulose. The
10 presence of biotin-labeled DNA wherein mismatched regions are bound to the nitrocellulose via MutS protein, is detected by visualizing the binding of streptavidin to biotin. See WO 95/12689. MutS protein has also been used in the detection of point mutations in a gel-mobility-shift assay (Lishanski, A. *et al.*, *Proc. Natl. Acad. Sci. USA* 91:2674-2678 (1994)).

15 Other methods, such as those described below, can be used to distinguish a FATP allele from a reference allele, once a particular allele has been characterized as to DNA sequence.

Allele-specific probes. The design and use of allele-specific probes for analyzing polymorphisms is described by e.g., Saiki *et al.*, *Nature* 324:163-166 (1986);
20 Dattagupta, EP 235,726, Saiki, WO 89/11548. Allele-specific probes can be designed so that they hybridize to a segment of a target DNA from one individual but do not hybridize to the corresponding segment from another individual due to the presence of different polymorphic forms in the respective segments from the two individuals. Hybridization conditions should be sufficiently stringent that there is a significant
25 difference in hybridization intensity between alleles, and preferably an essentially binary response, whereby a probe hybridizes to only one of the alleles. Some probes are designed to hybridize to a segment of target DNA such that the polymorphic site aligns with a central position (e.g., in a 15-mer at the 7 position; in a 16-mer, at either the 8 or

9 position) of the probe. This design of probe achieves good discrimination in hybridization between different allelic forms.

Allele-specific probes are often used in pairs, one member of a pair showing a perfect match to a reference form of a target sequence and the other member showing a perfect match to a variant form. Several pairs of probes can then be immobilized on the same support for simultaneous analysis of multiple polymorphisms within the same target sequence.

Allele-specific Primers. An allele-specific primer hybridizes to a site on target DNA overlapping a polymorphism, and only primes amplification of an allelic form to which the primer exhibits perfect complementarity. See Gibbs, *Nucleic Acid Res.* 17:2427-2448 (1989). This primer is used in conjunction with a second primer which hybridizes at a distal site. Amplification proceeds from the two primers, resulting in a detectable product which indicates the particular allelic form is present. A control is usually performed with a second pair of primers, one of which shows a single base mismatch at the polymorphic site and the other of which exhibits perfect complementarity to a distal site. The single-base mismatch prevents amplification and no detectable product is formed. The method works best when the mismatch is included in the 3'-most position of the oligonucleotide aligned with the polymorphism because this position is most destabilizing to elongation from the primer (see, e.g., WO 93/22456).

Gene Chips. Allelic variants can also be identified by hybridization to nucleic acids immobilized on solid supports (gene chips), as described, for example, in WO 95/11995 and U.S. Patent No. 5,143,854, both of which are incorporated herein by reference. WO 95/11995 describes subarrays that are optimized for detection of a characterized variant allele. Such a subarray contains probes designed to be complementary to a second reference sequence, which is an allelic variant of the first reference sequence.

The present method is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

Materials and Methods

5 The following Materials and Methods were used in the work described in Examples 1-5.

Sequence Alignment of FATP Clones. The DNA sequence for mouse FATP1 was obtained from the National Center for Biotechnology Information nonredundant database. cDNAs for mmFATP2, 3, 4, and 5 were obtained by screening mouse
10 expression libraries (purchased from GIBCO/BRL) with probes derived from the cloned expressed sequence tags (ESTs) (Research Genetics, Huntsville, AL). Full-length clones were obtained for mmFATP2 and 5 and partial sequences for mmFATP3 and 4. The sequences described herein have been deposited in the GenBank database (Accession Nos. FATP2, AF072760; FATP3, AF072759; FATP4, AF072758; FATP5,
15 AF072757).

Neither FATP2 nor FATP5 contains an in-frame stop codon upstream of the putative initiator methionine; initiator methionines were assigned by homology with that in mmFATP1 and by the presence of a signal sequence immediately after it. The *Mycobacterium tuberculosis*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*
20 sequences were present in the dbEST database as part of the sequencing projects for these organisms. Sequences were aligned utilizing a ClustalX algorithm and the resulting alignment exported to SeqVu. Homologous amino acid substitutions are boxed in Figure 1 and were determined using the Dayhoff 250 method with a 50% homology cutoff.

25 Cell Transfection and LCFA Uptake. COS cells were cotransfected using the DEAE-dextran method with the mammalian expression vector pCDNA 3.1 (Invitrogen) expressing the gene for CD2 (pCDNA-CD2) in combination with either a pCDNA 3.1 or pCMVSPORT2 (GIBCO/BRL) expression vector containing one of the murine or

nematode *FATP* genes (*pCDNA-mmFATP1*, *pCDNA-FATP2*, *pCMVSPORT-FATP5*, *pCDNA-ceFATPb*). Two days after transfection, cells were assayed for CD2 expression with a phycoerythrin-coupled anti-CD2(PE-CD2) monoclonal antibody (PharMingen), and fatty acid uptake was assayed with a BODIPY-labeled fatty acid analogue

5 (Molecular Probes). Briefly, cells were washed twice with PBS (phosphate buffered saline) and stained with PE-CD2 at 4°C for 30 min in PBS containing 10% fetal calf serum. They were then washed three times with PBS/fetal calf serum for 5 min followed by an incubation for 2 min at 37°C in fatty acid uptake solution, which contained 0.1 μ M BODIPY-FA and 0.1% fatty acid-free BSA (bovine serum albumin)

10 in PBS (Schaffer, J.E. & Lodish, H.F. (1994) *Cell* 79:427-436). After 2 min, the cells were washed four times with ice-cold PBS/0.1% BSA. The cells were then removed from the plates with PBS containing 5 mM EDTA and resuspended in PBS containing 10% fetal calf serum and 10 mM EDTA. PE-CD2 and BODIPY-FA fluorescence were measured using a FACScan (Becton Dickinson). COS cells were gated on forward

15 scatter (FSC) and side scatter (SS). Cells exhibiting more than 300 CD2 fluorescence units (dsim) representing 15% of all cells were deemed CD2 positive and their BODIPY-FA fluorescence was quantitated.

E. coli-Based LCFA Uptake Assay. The full-length coding region of mtFATP and a control protein, the mammalian transcription factor TFE3, were subcloned into the

20 inducible, prokaryotic expression vector pET (Novagen). Expression was induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 1 hour, or cells were left uninduced. Cells were washed in PBS/0.1% BSA and resuspended in 1 ml PBS/0.1% BSA containing 0.1 μ M [3 H]palmitate (NEN) at 37°C. Uptake was stopped after the indicated incubation time by transferring the cells onto filter paper using a cell harvester

25 (Brandel, Bethesda, MD). Filters were washed extensively with ice-cold PBS/0.1% BSA, and [3 H]palmitate was quantitated by scintillation counting.

Northern Blots. Northern blot analysis of murine FATP expression was done using poly(A) mRNA blots (Clontech). Probes of each of the FATPs were derived from the 3' untranslated regions of each gene and were <60% identical in sequence. Probes

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were labeled by random priming (Boehringer Mannheim) and hybridized at 65°C. Blots were extensively washed in 0.2% SSC/0.1% SDS at 65°C.

Generation of Phylogenetic Trees. Complete and partial sequences for *FATP* genes from human, rat, mouse, puffer fish, *Drosophila melanogaster*, *C. elegans*, *S. cerevisiae*, and *M. tuberculosis* were aligned using ClustalX. A homologous region of 48 amino acids (residues 472-519 in mmFATP1) from all of the genes was used to determine phylogenetic relationship within ClustalX. Based on these data a phylogenetic tree was generated using Tree View PPC (Figure 5).

Nomenclature. It is proposed that the *FATP* genes be given a species specific prefix (mm, *Mus musculus*; hs, *Homo sapiens*; mt, *M. tuberculosis*; dm, *D. melanogaster*; ce, *C. elegans*, sc, *S. cerevisiae*) and numbered such that mammalian homologues in different species share the same number but differ in their prefix. Since the two *C. elegans* genes cannot be paired with a specific human or mouse *FATP*, they have been designated *ceFATPa* and *ceFATPb*.

15 Example 1: Identification of Novel Mammalian FATPs

The National Center for Biotechnology Information EST database was screened, using the mouse *FATP* protein sequence (mmFATP1), to identify novel *FATPs*. This strategy led to the identification of more than 50 murine EST sequences which could be assembled into five distinct contiguous DNA sequences (contigs). One contig was identical to the previously cloned *FATP*, which has been renamed *FATP1*. Another, which has been renamed *FATP2*, is the murine homologue of a rat gene previously identified by others as a very long chain acyl-CoA synthase (Uchiyama, A., Aoyama, T., Kamijo, K., Uchida, Y., Kondo, N., Orii, T. & Hashimoto, T. (1996) *J. Biol. Chem.* 271:30360-30365). The other three contigs represented novel genes (*FATP3*, 4, and 5).

25 Full-length clones for *FATP2* and *FATP5* and nearly complete sequences for *FATP3* and 4 (Figure 1) were obtained by screening cDNA libraries made from mouse day 10.5 embryos and adult liver. Also identified were human homologues for each of the murine genes in the EST database. A sixth human gene was also identified; whether

this gene is also present in the mouse will require additional studies. Map positions are given in Tables 2 and 3.

The genetic loci for all of the human genes, with the exception of FATP5 which was already mapped as an unknown EST, were determined using the radiation hybrid
5 panels. The map positions given below show the distance (in centiRays) from the closest framework marker. As a guideline, there are approximately 300kb/cR.

Table 2. Mapping Data for Human Genes

	hsFATP1	Chromosome Chr19 places 13.35 cR from WI-6344 (lod>3.0)
10	hsFATP2	Chromosome Chr15 places 4.92 cR from D15S126 (lod>3.0)
	hsFATP3	Chromosome Chr1 places 13.24 cR from WI-2862 (lod>3.0)
15	hsFATP4	Chromosome Chr9 places 7.80 cR from WI-9685 (lod>3.0)
	hsFATP5	unknown EST previously mapped to near D19S418
	hsFATP6	Chromosome Chr5 places 1.41 cR from WI-4907 (lod>3.0)

The mouse map is an internal backcross panel consisting of 188 mouse
20 backcross DNA's plus 4 controls (B6, Spretus, F1, Water). The backcross was constructed by crossing B6 by Spretus animals and then crossing those F1's back to B6. Mapping is accomplished by taking advantage of recombinational events during meiosis, and the use of PCR primers to detect the differences (by size or re-annealing events) at any given locus between the B6 and Spretus allele.

25 For the purposes of mapping, a novel set of primers (gene of interest) is used to amplify from all 188 DNA's and then typed as being a B6 ("B") or a Spretus ("S"). This

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string of B's and S's is entered into the Map Manager program, which does a best fit calculation by comparing the string of 188 typings from the gene of interest to all loci already extant in the panel, for all 20 chromosomes. The gene of interest is then assigned to a particular area on a particular chromosome according to a number of
 5 parameters, including the minimalization of double cross-overs, and the highest LOD scores. Indicated in Table 3 are distances to the closest markers on either side of the FATP locus.

Table 3. Mapping Data for Mouse Genes

10	mmFATP1	Chromosome 8 places 2.82 cM from D8Mit132 (lod 43.4) and 1.81 cM from D8Mit74 (lod 43.5)
	mmFATP2	Chromosome 2 places 1.29 cM from D2Mit258 (lod 47.9) and 1.75 cM from D2NDS3 (lod 44.9)
15	mmFATP3	Chromosome 3 places 2.54 cM from D3Mit22 (lod 29.5) and 19.62 cM from D3Mit42 (lod 13.6)
20	mmFATP4	Chromosome 2 places 13.78 cM from D2Mit1 (lod 22.9) and 3.85 cM from D2Mit65 (lod 41.9)
	mmFATP5	Chromosome 7 places 7.28 cM proximal of D7Mit21 (lod 28.3)

Example 2: Assessment of Function

The ability of the newly identified mouse genes to function as fatty acid
 25 transporters was assessed using a fluorescence-activated cell sorting-based assay. COS cells were transiently cotransfected with expression vectors encoding the cell surface

protein CD2 and either mmFATP1, mmFATP2, or mmFATP5, respectively. Two days after transfection, COS cells were stained with an antibody to CD2 and then incubated with a BODIPY-labeled fatty acid [BODIPY-FA, (Schaffer, J.E. & Lodish, H.F. (1994) *Cell* 79:427-436)]. The cells were then washed extensively, lifted off the dish, and

5 analyzed by fluorescence-activated cell sorting. As judged by the number of CD2-positive cells, the transfection efficiency was approximately 20-30%. Fatty acid uptake was quantitated in the transiently transfected COS cells by measuring the BODIPY-FA fluorescence of the CD2-positive cells. Expression of CD2 had no effect on fatty acid uptake as shown by the finding that COS cells expressing only the transfected CD2

10 cDNA (CD2-positive) had the same low level of BODIPY-FA uptake as did untransfected (CD2-negative) control cells (Figure 2A, control). In COS cells cotransfected with CD2 and mmFATP1, mmFATP2, or mmFATP5, uptake of BODIPY-FA by the transfected (CD2-positive) cells was increased between 15- to 90-fold over control (CD2 cDNA only) cells (Figures 2A-2D).

15 Example 3: Expression Patterns of Murine FATPs

Expression patterns of members of the murine *FATP* gene family were characterized by Northern blot analysis; to avoid cross-hybridization, the probes used were from the 3' untranslated region of these genes, which are less than 60% identical in sequence. The expression pattern of FATP1 agrees with that previously found

20 (Schaffer, J.E. & Lodish, H.F. (1994) *Cell* 79:427-436). Here, expression was seen primarily in heart and kidney. FATP2 is expressed almost exclusively in liver and kidney, which corresponds to the reported tissue distribution of the rat homologue [very long chain acyl-CoA (VLACS)] as assessed by Western blotting (Uchiyama, A., Aoyama, T., Kamijo, K., Uchida, Y., Kondo, N., Orii, T. & Hashimoto, T. (1996) *J. Biol. Chem.* 271:30360-30365). FATP3 is present in lung, liver, and testis. FATP5 is

25 expressed only in liver and cannot be detected in other tissues even when the blot is overexposed. The human homologue of FATP5 is also liver specific and is not expressed in a wide array of other tissues tested, including fetal liver.

Example 4: FATPs Are Evolutionarily Conserved

The EST database was searched, using sequences conserved among the five murine FATP genes, for *FATP* genes in other organisms. Two homologues were found in *C. elegans* and one in *M. tuberculosis*. One of the *C. elegans* genes was cloned from a cDNA library and expressed in COS cells, as described for the murine FATPs. Overexpression of the nematode FATP resulted in a 15-fold increase of BODIPY-FA uptake compared with control cells (Figure 3). The mycobacterial *FATP* gene was isolated from a phage library and assessed for its ability to facilitate fatty acid uptake. *E. coli* transformed with a prokaryotic, isopropyl β -D-thiogalactoside-inducible expression vector containing the mycobacterial *FATP* gene demonstrated a significant increase in the rate of [3 H]palmitate uptake after induction, compared with uninduced bacteria or *E. coli* transformed with a control protein (Figure 4). Novel *FATP* genes were also identified in *F. rubripes* (puffer fish) and *D. melanogaster*.

Example 5: Phylogenetic Tree of FATPs

Faergeman *et al.* (Faergeman, N.J., DiRusso, C.C., Elberger, A., Knudsen, J. & Black, P. N. (1997) *J. Biol. Chem.* 272:8531-8538) identified three regions of very strong conservation between the *scFATP* and *mmFATP1* genes. The sequences of the FATPs were compared over a 311-amino acid FATP "signature sequence" which includes these conserved regions corresponding to amino acids 246-557 in mmFATP1 (underlined in Figure 1). When compared with the National Center for Biotechnology Information nonredundant database, only one region of the "FATP signature sequence" shows significant homology to other proteins. This small stretch of amino acids (underlined in Fig. 1) is an AMP-binding motif found in a multitude of other proteins, such as acyl-CoA synthase, several CoA lipases, and gramicidin S synthetase component II (Schaffer, J.E. & Lodish, H.F. (1994) *Cell* 79:427-436). The relevance of this motif to fatty acid transport is unclear. Other highly conserved regions among the FATPs, including long stretches of amino acids >90% identical from mycobacteria to humans, are not found in any other class of proteins. A 48-amino acid segment of the

FATP signature sequence was used to construct a phylogenetic tree (Figure 5). Each of the human and mouse genes form their own branch; hsFATP6, which as yet has no murine homologue, is most closely related to hsFATP3 and mmFATP3. As expected, mVLACS is closer in sequence to mmFATP2 than to hsFATP2. The *FATP* genes of
5 invertebrates i.e., *C. elegans* and *D. melanogaster*, are most closely related to each other. Surprisingly, the mycobacterial gene is more closely related to the human and mouse *FATP5* genes than to the FATPs of any of the lower organisms. Whether this reflects coevolution of the mycobacterial and human genes awaits further study.

Materials and Methods

10 The following materials and methods were used in the work described in Examples 6-10.

Isolation of full-length human FATP1 and 4

Full-length clones encoding human FATP1 and human FATP4 were identified by searching databases for sequences similar to murine FATP1-5 coding regions using
15 the BlastX algorithm (Altschul *et al.*, *J. Mol. Biol.* 215: 403-410, 1990).

A concatamer of nucleotide sequences comprising the coding sequences of mmFATP1 (Genbank Accession U15976), mmFATP2, mmFATP3 (SEQ ID NO:6), mmFATP4 (SEQ ID NO:8) and mmFATP5 (SEQ ID NO:10) was used to search the Millennium database using the BLASTX algorithm. Sequences with a score >150 were
20 evaluated for whether they represented known FATP coding sequences.

Human clones with similarity to the 5' end of murine FATP sequences were sequenced completely. Clones encoding full-length human FATP1 were obtained from a heart cDNA library constructed in the mammalian expression vector pMET7 (Tartaglia *et al.*, *Cell*, 83: 1263-1271, 1995). Clones encoding full-length human
25 FATP4 were obtained from a spleen cDNA library constructed in the mammalian expression vector pMET7.

5

Sequences were aligned with the DNASTar program using the Clustal method. Hydrophobicity plots were generated with DNA Strider using the Kyte Doolittle method.

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NBT-2 photoemulsion and exposing for 7 days at 4°C, followed by development with Kodak Dektol developer. Slides were counter stained with haematoxylin and eosin and photographed. Controls for the in situ hybridization experiments include the use of a sense probe which showed no signal above background in all cases.

5 Northern Blotting

Human mRNA blots were obtained from Invitrogen or Clontech. PCR fragments from the 3' untranslated regions of human FATPs were used as probes. Blots were probed with ³²P-labeled DNA probes using the Rapid-Hyb buffer (Amersham) according to the manufacturer's instructions.

- 10 Cell transfection and LCFA uptake. COS cells were cotransfected, using lipofectamine (GIBCO BRL) according to the manufacturer's instructions, with the mammalian expression vector pCDNA3.1 (Invitrogen) expressing the gene for CD2 in combination with a pMET7 expression vector (Tartaglia *et al.*, *Cell*, 83:1263-1271, 1995) containing hsFATP1 (pMET7-hsFATP1) or hsFATP4 (pMET7-hsFATP4) or
- 15 pMET7 alone. Two days after transfection, cells were assayed for CD2 expression with a phycoerythrin-coupled anti-CD2 (PE-CD2) monoclonal antibody (PharMingen), and fatty acid uptake was assayed with a BODIPY-labeled fatty acid analog (Molecular Probes) as described above.

Example 6: Determination of Expression of mmFATPs

- 20 mmFATP4, and to lesser extent mmFATP2, are expressed at high levels in the brush border layer of the small intestine.

- Cell transfection and LCFA uptake. COS cells were cotransfected, using lipofectamine (GIBCO BRL) according to the manufacturer's instructions, with the mammalian expression vector pCDNA3.1 (Invitrogen) expressing the gene for CD2 in
- 25 combination with a pMET7 expression vector (Tartaglia *et al.*, *Cell*, 83:1263-1271, 1995) containing hsFATP1 (pMET7-hsFATP1) or hsFATP4 (pMET7-hsFATP4) or pMET7 alone. Two days after transfection, cells were assayed for CD2 expression with

a phycoerythrin-coupled anti-CD2 (PE-CD2) monoclonal antibody (PharMingen), and fatty acid uptake was assayed with a BODIPY-labeled fatty acid analog (Molecular Probes) as described above.

Absorption of dietary fat requires transport of free fatty acids across the apical
5 membrane of epithelial cells in the small intestine. Previous studies suggested that this transport is protein-mediated; however, the transport protein had not yet been identified. In situ hybridization was performed on each of the three regions of the small intestine -- duodenum, jejunum and ileum -- as well as the colon, using probes from the 3' untranslated regions of mmFATP1, mmFATP2, mmFATP3, mmFATP4 and
10 mmFATP5, to determine whether any of the mouse FATPs are expressed in the small intestine. It was expected that a protein involved in fatty acid absorption would be expressed in the epithelial cells of the small intestine, but absent from the colon.

Expression of mmFATPs in the jejunum was identical to that in the ileum in all cases. High levels of mmFATP4 mRNA were present in the epithelial cells of the
15 jejunum and ileum, and lower, but significant, amounts were detected in the epithelial cells of the duodenum. Significantly, FATP4 mRNA was absent from other cell types of the small intestine and no FATP4 mRNA could be detected in any of the cells of the colon. FATP2 mRNA was present in the epithelial cells of the duodenum at a level similar to that of FATP4, but was present at lower levels in the jejunum and ileum. No
20 signals above background were detected for mmFATP1, mmFATP3 and mmFATP5 in any of the intestinal tissues. mmFATP3 and FATP5 were clearly detectable by in situ hybridization in adult liver and mmFATP1 could be detected in a variety of tissues on a whole embryo in situ, indicating that the FATP1, 3, and 5 probes were working.

mmFATP4 expression is predominant in the small intestine compared to the
25 other organs of the mouse embryo. In the small intestine, FATP4 expression is limited to differentiated enterocytes, while no signal is detected in the connective tissue or the undifferentiated epithelial cells in the crypts. Differentiated enterocytes are known to be the cells that mediate the uptake of fatty acids. FATP4 is specifically and strongly expressed in the epithelial cells of adult murine duodenum and ileum but not colon.

Other FATPs, such as FATP5, are not expressed in the small intestine. Thus, FATP4 is the major FATP in the mouse small intestine. Given its high level of expression, it is likely that FATP4, and to a lesser extent FATP2, play an important role in the absorption of fatty acids.

5 mmFATP2, and mmFATP5 are expressed in hepatocytes

Northern analysis of mmFATP2, mmFATP3, mmFATP4 and mmFATP5 showed expression in the liver. To determine whether these proteins are present in hepatocytes or other cells types present in liver homogenates, in situ hybridizations were performed. mmFATP2, and mmFATP5 mRNA was clearly present in
10 hepatocytes, and was not concentrated in other cell types such as endothelial cells or macrophages. No signal above background was detected for mmFATP1 in any of the cell types in the liver, consistent with the results of the Northern blotting.

Example 7: Isolation and Sequence Analysis of Full-length Human FATP1 and Full-length Human FATP4

15 To identify human cDNA clones encoding FATP family members, Millennium databases were searched for sequences similar to murine FATP1-5 coding regions. Two clones were analyzed in detail; inspection of the entire DNA sequence of these two clones showed that they encode the human orthologs of mmFATP1 and mm FATP4, respectively. These two clones were designated hsFATP1 and hsFATP4, and their
20 DNA and predicted protein sequences are shown in Figures 44A-44C and 45, and 50A-50C and 51. hsFATP1 is predicted to encode a 646 amino acid, 71 kD protein with multiple membrane-spanning domains (Figure 28A). HsFATP4 is predicted to encode a 643 amino acid, 72 kD protein with multiple membrane spanning domains (See Figure 29A). A comparison of the DNA sequences of mouse and human FATP1 and mouse
25 and human FATP4 (Figures 30A-30B and 31A-31B) shows that the mouse and human orthologs are 85% (FATP1) and 87% (FATP4) identical to each other within the coding sequences given in these figures. At the amino acid level, hsFATP1 and hsFATP4 are

~90% identical to their respective mouse orthologs within the coding region shown in these figures (Figures 32 and 33). The sequence identities between mouse and human FATP1 and FATP4 are considerably higher than the ones observed between different FATP family members within one species (~40%-60%) and are present in the N-terminal part of the protein, a region that is poorly conserved between different FATP family members. This high degree of sequence conservation clearly demonstrates that the newly identified human FATPs are orthologs of mouse FATP1 and FATP4 rather than novel FATP family members.

Table 4 is an identity/similarity matrix comparing the amino acid sequences of FATP1 and 4 from human and mouse. This shows that the gene whose sequence is shown in Figure 43A is indeed human FATP4, since it is 91% identical with the murine FATP4 but only 62% identical with the closest related human FATP, which is FATP1.

Table 4				
Identity/Similarity Matrix				
	hsFATP4	mmFATP4	hsFATP1	mmFATP1
hsFATP4	---	93.2	72.3	72.0
mmFATP4	91.0	---	71.2	71.1
hsFATP1	61.9	61.0	---	92.4
mmFATP1	60.7	59.6	89.5	---

Example 8: Isolation and Sequence Analysis of Full-length Human FATP6

A search of EST databases identified a set of overlapping human sequences that were similar to FATPs, but did not have a clear mouse ortholog. One of these EST clones was found to encode a full-length cDNA. The entire insert of this clone was sequenced and designated hsFATP6. The DNA and predicted protein sequences of hsFATP6 are shown in Figures 54A-54C and 55. HsFATP6 is predicted to encode a 619 amino acid, 70 kD protein with multiple membrane-spanning domains (Figure



The tissue distribution of human FATPs was assessed by Northern blotting.

Human FATP3 was expressed in a large variety of tissues. In contrast, human FATP5 was present at high levels in the liver, but was undetectable in all other tissues examined. Thus, both hsFATP3 and hsFATP5 recapitulate the expression pattern of their mouse orthologs (see above). HsFATP6 is a novel FATP with no mouse ortholog as yet. Northern blotting shows that hsFATP6 is expressed at high levels in the heart, but is undetectable in other tissues, including skeletal and smooth muscle. This tissue distribution suggests that human FATP6 performs an important role in energy metabolism in the heart; blocking FATP6-mediated fatty acid transport may therefore be beneficial for a number of heart diseases, e.g., ischemic heart disease.

To identify the major FATP expressed in the human small intestine, Northern blotting was performed on a blot containing mRNA from human stomach, jejunum, ileum, colon, rectum and lung. hsFATP5 and hsFATP6 were undetectable in any of these tissues. FATP5 is only expressed in liver and FATP6 only in heart. hsFATP2 was weakly expressed in the colon, and an even weaker signal was detectable in jejunum, ileum and lung lanes. hsFATP3 was expressed well in the lung, but was only weakly expressed in the other tissues tested. Importantly, no difference was seen in the expression of hsFATP3 between small intestine and stomach or colon, suggesting that the expression observed is not related to fatty acid absorption in the small intestine. hsFATP4 was clearly expressed in both jejunum and ileum; expression was

significantly lower in the colon and was absent in the stomach. This expression pattern is consistent with a major role for FATP4 in absorption of fatty acids in the human gut.

Example 10: Expression of hsFATP1 and hsFATP4 Promotes Transport of Fatty Acids

COS cells were cotransfected using lipofectamine with the mammalian
5 expression vector pCDNA-CD2 in combination with one of the FATP-containing
expression vectors (pMET7-hsFATP1 or pMET7-hsFATP4) or an insertless expression
vector (pMET7, control) as described in Materials and Methods for Examples 6-10.
COS cells were gated on forward scatter and side scatter. Cells exhibiting more than
400 CD2 fluorescence units representing ~30% of all cells were deemed CD2-positive.
10 The percent of CD2-positive cells exhibiting a BODIPY-fluorescence of >300 is plotted
for the three different vectors tested (Figure 37).

Example 11: Stable Expression of Human FATP4 in 293 Cells

Stable cell lines were generated as follows. A DNA fragment containing the
entire hsFATP4 coding sequence as well as 100 nucleotides of 5' and 50 nucleotides of
15 3' untranslated region was inserted into the vector pIRES-neo (Clontech) using standard
cloning techniques. The resulting construct or a vector control (pIRES-neo) was
transfected into 293 cells using the lipofectamine method (Gibco BRL) according to the
manufacturer's directions. Cells that had taken up the DNA were selected with 1 mg/ml
G418 (Gibco BRL). Single colonies were picked 1 to 2 weeks after transfection and
20 grown in medium containing 0.8 mg/ml G418. Colonies were screened for the ability to
take up fatty acids by measuring uptake of a fluorescently labeled fatty acid (BODIPY-
FA). About 40 colonies transfected with the pIRES-neo containing FATP4 and ~20
colonies transfected with pIRES-neo control were analyzed. All 20 of the vector control
clones showed amounts of BODIFY-FA uptake similar to each other and to
25 untransfected 293 cells. In contrast, among the 40 FATP4 transfected clones, 3 had a 5-
to 10-fold increased BODIPY-FA uptake compared to any of the vector controls, and a
large number (~20) showed an approximately two-fold increase in BODIPY-FA levels.

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This distribution is consistent with FATP4 conferring increased fatty acid uptake in these cells. One of the cell lines with the highest amount of BODIPY-FA uptake was selected to be used for measuring uptake of tritiated fatty acid.

The uptake of tritiated oleate over time by either FATP4 expressing or control
5 cells was assayed over time. Expression of FATP4 increases the rate of fatty acid uptake by over 3-fold, demonstrating that FATP4 is, like the other FATPs, a functional fatty acid transporter (Figure 38).

Example 12: Immuno-staining with FATP4-Specific Antiserum

A polyclonal antiserum against the C-terminus of mmFATP4 was raised using a
10 GST-fusion protein having mmFATP4-specific amino acid sequence 552-643 (AVASP...GEEKL). In western blot experiments, the purified antibody reacted strongly with a synthetic peptide matching the C-terminus of mmFATP4, but not with a corresponding region of mmFATP2, mmFATP3, or mmFATP5. The mmFATP4 specific polyclonal antiserum detects, in western blot experiments with enterocyte
15 lysates from 3 different mice, a ~70 kDa protein, which is in accordance with mmFATP4's predicted molecular weight of 72 kDa. The binding is specific for mmFATP4, since it can be completely abolished by preincubation of the antiserum with the GST-fusion peptide used to raise the antibody.

Immunofluorescence experiments were performed using the anti-mmFATP4
20 antiserum on fresh frozen sections of murine small intestine. The antibody binding demonstrates strong expression of mmFATP4 in enterocytes, confirming the results of the in situ hybridization experiments. At higher magnifications it is apparent that mmFATP4 is expressed at the apical side of the enterocyte, indicating that the transporter is present in the brush border membrane, which is known to mediate the
25 uptake of fatty acids from the intestinal lumen.

Immuno-electron microscopy studies were performed on fresh frozen murine intestinal cells. The gold particles used, appearing as black specks on the electron micrographs, indicate the subcellular localization of mmFATP4 to be on the microvilli

of the enterocyte. It can be seen from electron micrographs that mmFATP4 is localized exclusively in membranes, preferentially the apical plasma membrane, confirming that it is indeed a membrane protein.

Methods for Immunofluorescence and Immunogold Electron Microscopy

5 Unfixed mouse small intestine was washed with Hank's buffered salt solution containing 1 mM EDTA, infused with 2.3 M sucrose solution, and embedded in O.C.T., 4583 compound. The material was thick sectioned (15 μ M - 40 μ M). The sections were washed in PBS containing 1% BSA and 0.075% glycine to block non-specific binding. Primary and secondary antibodies were diluted in PBS with 10% FCS and
10 incubated for 1h. The sections were mounted in 90% glycerol/PBS containing 1 mg/ml paraphenylenediamine, and examined with a Bio-Rad MRC 600 confocal, mounted on a Zeiss Axioscop.

 For the immunogold labeling, the tissue was fixed with 2% paraformaldehyde in PBS for 10 minutes, after which it was cryoprotected by infiltration with 2.3 M sucrose
15 in 0.1 M phosphate buffer (pH 7.4) containing 20% polyvinylpyrrolidone, and then mounted on aluminum cryo nails and frozen in liquid nitrogen (Tokuyasu, K.T., *J. Microscop.* 143:139-149, 1986). Ultrathin sections were collected on carbon/formvar-coated nickel grids. The primary antibody (anti-FATP4) was diluted in 10% FCS in PBS and incubated overnight at 4 C, followed by donkey anti-rabbit IgG-gold (12 nm)
20 (Jackson Labs) for 1h. The sections were stained in 2% neutral uranyl acetate (20 minutes) and absorption stained with 2% uranyl acetate in 0.2% methylcellulose containing 3.2% polyvinyl alcohol. The sections were examined with a Philips EM 410 electron microscope.

Example 13: Inhibition of Fatty Acid Uptake Specific to FATP4 Demonstrated in
25 Isolated Mouse Enterocytes

 Phosphorothioate derivatives of the following oligonucleotides were synthesized:



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uptake, but not a control oligonucleotide differing only in the sequence but not the nucleotide content, ruling out a toxic or otherwise nonspecific inhibitory effect of this oligonucleotide due to its chemical composition.

As a further control experiment, the uptake of oleate was measured along with the uptake of methionine in the same cultured enterocytes. Antisense oligonucleotide, mismatch sequence oligonucleotide, or no oligonucleotide was added to a concentration of 100 μ M to cultures of enterocytes. After incubation for 48 hours, the uptake of both 3 H-labeled oleate and 35 S-labeled methionine was assayed. Results are shown in Figure 42. Fatty acid uptake is at the left side of the paired bars; methionine uptake is on the right side of the paired bars. The fact that amino acid uptake was not influenced by the antisense oligonucleotide treatment further supports the conclusion that the antisense oligonucleotide causes a specific reduction in translation of FATP4-specific mRNA.

Example 14: mmFATP2 Is Expressed in Proximal Renal Tubule Epithelium

Northern analysis showed that mmFATP1, mmFATP2, and mmFATP4 are present in the kidney. In situ hybridization (methods as for Example 6) was performed to determine which cell type(s) of the kidney these mRNAs are expressed in. mmFATP1 mRNA was present in virtually all cells throughout the kidney with no obvious preference for a particular cell type. In contrast, mmFATP2 was expressed only in the renal cortex. Within the cortex, expression of mmFATP2 was restricted to the epithelial cells of the proximal renal tubules. The primary function of proximal renal tubule cells is the reabsorption of filtered salts and nutrients (e.g., glucose), a process that requires mitochondrial oxidation and that can utilize fatty acids as energy substrates. Based on the localization of mmFATP2, it is possible that mmFATP2 is important for reabsorption in the kidney by allowing uptake of an energy source (fatty acids) from the blood into renal epithelial cells. Alternatively, if fatty acids need to be reabsorbed in the kidney, similarly to glucose, FATP2 could be involved in the reabsorption of fatty acids. Determination of the subcellular localization of FATP2 will distinguish between these two possibilities.

Table 5. Mouse FATP mRNA Expression

Mouse Probes	mFATP1	mFATP2	mFATP3	mFATP4	mFATP5
E18.5 embryo expression	everywhere, brain = thymus> heart> brown fat, others	liver (hepatocytes)	-	Brain, small intestine, superior cervical ganglion (SCG), dorsal root ganglion (DRG), other regions have lower expression	Mouse Probes
Duodenum	-	villi (surface epithelium)	-	villi (surface epithelium)	-
Jejunum	-	villi (surface epithelium)	-	villi (surface epithelium)	-
Ileum	-	villi (surface epithelium)	-	villi (surface epithelium)	-
Colon	low expression in the crypt	very low level in the crypt	-	-	-
Kidney	cortex and medulla	proximal tubules	-	-	-

Table 5 (continued). Mouse FATP mRNA Expression

Mouse Probes	mFATP1	mFATP2	mFATP3	mFATP4	mFATP5
Liver	-	hepatocytes	hepatocytes	-	hepatocytes
Pancreas	exocrine secretory units or acinar cells; endocrine pancreas (islet) are negative	exocrine secretory units or acinar cells; endocrine pancreas (islet) are negative	-	-	-
Brain	Neuronal expression throughout the brain including hypothalamus	-	-	Neuronal expression throughout the brain including hypothalamus	-
Heart	myocytes	-	-		
Testis	seminiferous tubules	-	seminiferous tubules		
Lung	bronchiole	-	-		
Adipose	adipocyte	adipocyte	-		

Example 15: Isolation of full-length human FATP3

Full-length clones encoding human FATP3 were identified by searching databases for sequences similar to the murine FATP1-5 coding regions using the BlastX algorithm (Altschul *et al.*, *J. Mol. Biol.* 215: 403-410, 1990). Human clones with
5 similarity to the 5' end of murine FATP sequences were sequenced completely. A clone encoding full-length human FATP3 was obtained from a human bone library constructed in the mammalian expression vector pMET7 (Tartaglia, L.A. *et al.*, *Cell* 83: 1263-1271, 1995). To identify human cDNA clones encoding FATP family members, databases were searched for sequences similar to murine FATP1-5 coding regions. One
10 clone was found to encode the human ortholog of mmFATP3 and was designated hsFATP3. The DNA and predicted protein sequences of hsFATP3 are shown in Figures 94A and 94B. hsFATP5 is predicted to encode a 703 amino acid 75.6 kD protein with multiple membrane-spanning domains. A comparison of the DNA sequences of mouse and human FATP3 shows that the mouse and human orthologs are 81% identical to
15 each other within the coding region. At the amino acid level, hsFATP3 is ~86% identical to mm FATP3 within the coding region. The sequence identities between mouse and human FATP3 are considerably higher than those observed between different FATP family members within one species (~40%) and are present in the N-terminal part of the protein, a region that is poorly conserved between different FATP
20 family members.

Example 16: Substrate Specificity of Fatty Acid Transport in hsFATP-Transfected Clones

Using a mammalian expression vector, we generated 40 stable 239 cell lines expressing hsFATP4 and 20 cell lines transfected with a control plasmid. The ability of
25 the different cell lines to take up FA, as assessed by uptake assays using the fluorescently labeled Bodipy-palmitate, correlated well with their FATP4 expression levels determined by Western blotting (FIG. 95). All 20 vector control clones showed amounts of Bodipy-FA uptake similar to each other and to untransfected 239 cells. In contrast, among the 40 FATP4 transfected clones, a large number (~20) showed an

approximately 2-fold increase in Bodipy-FA uptake compared to any of the vector controls, and three had a 5- to 10-fold increase in Bodipy-FA uptake.

Several of the cell lines with the highest amount of Bodipy-FA uptake as well as isolated primary enterocytes were used to measure the uptake of radiolabeled FAs.

- 5 Short-term uptake by 293 cells and enterocytes of all FAs tested was linear (FIG. 97). hsFATP4 expression enhanced the rate of palmitate uptake approximately 3 fold over 293 cells transfected with vector alone (FIG. 97) and also accelerated the uptake of oleate but not of linolate, arachidonate, octanoate, butyrate or cholesterol (Table 6). Isolated primary enterocytes showed a similar preference for palmitate and oleate, and
10 absence of transport of arachidonate, octanoate, and butyrate, but displayed a more robust transport of linolate and cholesterol than the transfected 293 cells.

- To further characterize the substrate specificity of FATP4, we measured the uptake by stably transfected 293 cells of 5 μ M Bodipy-FA in the presence of a 20 fold molar excess (i.e., 100 μ M) of FAs, FA-derivatives and lipid soluble vitamins and
15 hormones. Both saturated and non-saturated fatty acids containing 10 to 26 C atoms strongly competed for uptake of Bodipy-palmitate (FIG. 96 and Table 7) and thus are presumed to be substrates of FATP4. In contrast, fatty acids with eight or fewer C atoms did not compete and thus are presumed not to be FATP4 substrates. Similarly, esters of long chain FAs and other hydrophobic molecules tested had no effect on
20 uptake of Bodipy-palmitate.

LCFA Uptake Assays (Methods)

- Bodipy-FA uptake assays using FACS were performed, adapted to a 96-well format. LCFA uptake assays with enterocytes or with stably transfected 293 cells were done as follows. Mixed micelles of radiolabeled FA (NEN) and taurocholate (Sigma) in
25 HBS were generated by brief sonication at 37°C. Equal volumes of cells and micelle solution were mixed, resulting in a final FA concentration of 25 μ M for antisense assays and 10 μ M for substrate specificity assays. Final taurocholate concentration was 5 mM. Cells were incubated for the indicated amount of time at 37°C. The assay was stopped by transferring the cells onto filter paper followed by extensive washes with

ice-cold HBS containing 0.1% BSA using a cell harvester (Brandell). Incorporated oleate was then determined by β -scintillation counting (Beckman).

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Table 6

Uptake of Different Substrates by FATP4 Expressing Cell Lines and Enterocytes

Fatty Acid	293 Cells Control*	293 Cells Stably Expressing FATP4	FATP4 specific	Enterocytes*
Palmitate	564	1695	1131	3036
Oleate	662	1122	459	117
Linolate	640	673	33	116
Arachidonate	3	5	2	0
Octanoate	0	0	0	5
Butyrate	0	50	50	73
Cholesterol	319	345	26	531

Uptake of different substrates by enterocytes and by control and stable FATP4-expressing 293 cells. The rates of uptake for the indicated fatty acids was measured over 4 min taking measurements every 30 s. All fatty acids were at a concentration of 10 μ M in HBS containing 5 mM taurocholate.

*Uptake measured as *pmol/min 10⁶ cells*

Table 7

Competition of Bodipy-FA Uptake by FATP4 Expressing Cells

Fatty Acids	Formula	Competition
Butyric Acid	$C_4H_8O_2$	-
Caproic Acid	$C_6H_{12}O_2$	-
Caprylic Acid	$C_8H_{16}O_2$	-
Capric Acid	$C_{10}H_{20}O_2$	++
Lauric Acid	$C_{12}H_{24}O_2$	++
Myristic Acid	$C_{14}H_{28}O_2$	++
Palmitic Acid	$C_{16}H_{32}O_2$	++
Stearic Acid	$C_{18}H_{36}O_2$	+
Oleic Acid	$C_{18}H_{34}O_2$	++
Linoleic Acid	$C_{18}H_{32}O_2$	++
Arachidic Acid	$C_{20}H_{40}O_2$	++
Lignoceric Acid	$C_{24}H_{48}O_2$	++
Cerotic Acid	$C_{26}H_{52}O_2$	++

Fatty Acid Derivatives

Fatty Acids	Formula	Competition
Palmitic Acid Methyl Ester	$C_{17}H_{34}O_2$	-
Stearic Acid Methyl Ester	$C_{19}H_{38}O_2$	-
Oleic Acid Ethyl Ester	$C_{20}H_{38}O_2$	-
Oleic Acid Oley Ester	$C_{36}H_{68}O_2$	-
Oleoyle CoA	$C_{39}H_{68}N_7O_{17}P_3S$	-
Cholesteryl Oleate	$C_{45}H_{78}O_2$	-

Table 7 Continued

Competition of Bodipy-FA Uptake by FATP4 Expressing Cells

Lipid-Soluble Vitamins & Homones

	Fatty Acids	Formula	Competition
5	Retinoic Acid (Pro-Vitamin A)	$C_{20}H_{28}O_2$	±
	Ergocalciferol (Vitamin D2)	$C_{28}H_{44}O_2$	-
	Tocopherol (Vitamin E)	$C_{29}H_{50}O_2$	-
	3-Phytylamenadione (Vitamin K1)	$C_{31}H_{46}O_2$	-
10	Prostaglandin E2	$C_{20}H_{32}O_5$	-

Competition for Bodipy-FA uptake by FATP4 expressing cells by different hydrophobic compounds. The uptake of 5 μ M Bodipy-FA, C1-Bodipy-C12 was measured in the presence of a 20-fold molar excess (i.e., 100 μ M) of the indicated fatty acids or fatty acid derivatives. The maximal 100% inhibition was defined as the amount of Bodipy-FA incorporated in the presence of 200 μ M lauric acid which was on average 18% \pm 5% that of untreated cells.

- : 0% - 30% inhibition by the indicated substance
- ±: 30% - 50% inhibition
- 20 +: 50% - 70% inhibition
- ++: 70% - 100% inhibition

All references cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

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